Effects of ambient temperature on feeding by herbivorous marsupials

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Declaration

This thesis contains published work and work prepared for publication that has been co-authored with collaborating researchers. All the data presented in this work is original research and the contribution of each co-author is stated below. No part of this thesis has been submitted for any previous degree. The term "we" is used to acknowledge co-authors in Chapters 2-6 as they are prepared for publication. The term "I" is used in the Introduction and Synthesis sections.

Chapter 1. A hot lunch for herbivores: physiological effects of elevated temperatures on mammalian feeding ecology

Authors: Phillipa K Beale, Karen J Marsh, William J Foley, Ben D Moore

Literature reviewed by Phillipa K Beale. Original manuscript written by Phillipa K Beale. All authors contributed ideas, edited and improved the manuscript.

Chapter 2. Reduced hepatic detoxification in marsupial herbivores following moderate heat exposure

Authors: Phillipa K Beale, Patrice K Connors, Karen J Marsh, M Denise Dearing, William J Foley

Experimental idea conceived by all authors, and carried out by Phillipa K Beale and Patrice K Connors. Manuscript written by Phillipa K Beale, other authors contributed ideas, edited and improved the manuscript.

Chapter 3. Changes in ambient temperature can be as important as plant secondary metabolites in limiting feeding in mammalian herbivore

Authors: Phillipa K Beale, Karen J Marsh, Ben D Moore, Andrew K Krockenberger, William J Foley

Experiments carried out by Phillipa K Beale. Respirometry experimental design and interpretation of results with help from Andrew K Krockenberger. Feeding trial experimental design with help from Karen J Marsh, Ben D Moore, and William J Foley. Nutritional analysis conducted by Phillipa K Beale, Karen J Marsh, and Ben D Moore. Manuscript written by Phillipa K Beale and edited and improved by other authors.

Chapter 4. Macronutrient selection changes with ambient temperature in a mammalian herbivore

Authors: Phillipa K Beale, Karen J Marsh, Ben D Moore, William J Foley

Experimental idea and design conceived by Phillipa K Beale. Nutritional analyses carried out by Phillipa K Beale, Karen J Marsh, and Ben D Moore. Manuscript written by Phillipa K Beale and edited and improved by all authors.

Chapter 5. Daily variation in ambient temperatures affect the fine scale feeding decisions of wild mammalian browsers

Authors: Phillipa K Beale, Karen J Marsh, Ben D Moore, William J Foley

Idea conceived by Phillipa K Beale and Karen J Marsh. Data analysed and manuscript written by Phillipa K Beale with significant contribution from other authors.

Chapter 6. Can plant secondary metabolites act as mitochondrial uncouplers? Implications for heat balance in animals

Authors: Phillipa K Beale, Cara Timpani, Karen J Marsh, Ben D Moore, William J Foley, Emma Rybalka

Idea conceived by Phillipa K Beale, Karen J Marsh, Ben D Moore, and William J Foley. Experimental work conducted by Phillipa K Beale, Cara Timpani and Emma Rybalka with significant technical expertise contributed by Cara Timpani and Emma Rybalka. Phillipa K Beale analysed the data and wrote the manuscript. Karen J Marsh, Ben D Moore, and William J Foley edited and improved the manuscript.

Phillipa K Beale

Signature

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All work was approved by the Australian National University Animal Experimentation Ethics Committee and conforms to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Protocol A2015/24, A2017/3). All plants and animals were collected under Section 273 of the Nature Conservation Act 2014 (License numbers: K9652, PL201689, LT2017936, PL201547, LT2015815, LT2016883).

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Abstract

In this thesis I show that the nutritional decisions of herbivores are inextricably tied to the thermal environment they experience. This pertains not just to the total energy eaten, but also to the macronutrients from which they choose to get that energy, and to the plant secondary metabolites (PSMs) that complicate the decision. I have comprehensively reviewed the literature to show that the relationship between thermal physiology and nutritional ecology runs deeper than simply adjusting food intake to meet changes in metabolic rate. Ties between thermoregulation and nutrition in endothermic herbivores includes the thermogenic nature of PSM metabolism, PSM interference with thermosensing, potential mitochondrial uncoupling by PSMs, energy wasting by heat generation, and simple obligatory diet induced thermogenesis. I then present results on whether temperature-dependent toxicity applies to marsupial folivores ingesting diets with PSMs using captive feeding studies in common brushtail possums (*Trichosurus vulpecula*) and ringtail possums (*Pseudocheirus peregrinus*). I show that when exposed to different ambient temperatures (10°C, 18°C, 26°C) overnight there is no difference in intake of high-PSM diets when fed at different temperatures. However, if given one week of exposure time both brushtail and ringtail possums decrease intake at warmer ambient temperatures. I consider this pattern consistent with temperature dependent toxicity, however it differs slightly from previous literature, a topic I discuss. A mechanism put forward by others for temperature-dependent toxicity is the down regulation of hepatic enzymes in warmer conditions. I test whether this may be the underlying mechanism in possums by conducting a sleep-time assay using an hepatically metabolised anaesthetic agent as a proxy for PSM metabolism. I show that the rate of hepatic metabolism by possums is slower following one week of exposure to warm ambient temperatures (26°C vs 10°C), while there is no difference following overnight exposure. This supports the previous work showing that TDT may be a result of down regulation of enzymes responsible for PSM metabolism. Following this I demonstrate using nutritional geometry that ambient temperature may influence the mixing of macronutrients to minimise heat generation. Brushtail possums chose to eat a diet lower in the more thermogenic macronutrient, protein at 26°C, compared to possums fed at cooler temperatures (10°C, 18°C). The addition of a PSM (cineole) however removed this effect, so it may be that ingesting

enough protein to deal with the protein cost of PSM ingestion overrides the desire to minimise the obligatory thermogenesis of a higher protein diet. I then consider data collected using radio collars fitted with microphones to determine how intake patterns are influenced by temperature and PSMs influence daily and per meal food intake. Semi wild koalas (*Phascolarctos cinereus*) ingest 6g less overall per 1°C increase in mean ambient temperature. This was due to a change in meal size rather than a change in meal number. In particular meal size was reduced by warm temperatures when leaves contain higher concentrations of a particular groups of PSMs (formylated phloroglucinol compounds). Following this I investigate whether there is evidence of mitochondrial uncoupling in cell-culture by a range of PSMs found in *Eucalyptus* leaves, and while I find some interesting results, I do not see any evidence for strong uncoupling, although this may be due to experimental design. Taken together the results in this thesis show that at scales from the cell to the animal in its environment, ambient temperature can have considerable impact on nutrition, and that warming ambient temperatures due to climate change are likely to result in new challenges for marsupial folivores, as well as other herbivore species ingesting PSM rich diets.

Introduction

Background

Climate change is continuing, with global mean surface temperature (GMST) having already risen by 1°C since mass industrialization. A recent report by the Intergovernmental Panel on Climate Change called for urgent international cooperation to halt the rise in GMST at a level below 1.5°C (Hoegh-Guldberg *et al.* 2018). However, already no person below the age of 32 residing in Australia has experienced a cooler than average month and extreme climatic events such as droughts and heat waves are increasing in frequency and severity. This exposes endothermic animals to increased risks of hyperthermia. At high temperatures, dissipation of heat generated in the body is more difficult. The idea that the rate at which heat can be dissipated into the environment may limit the rate at which heat generating physiological processes can take place, has recently gained some traction (Speakman & Król 2010). Coined "heat-dissipation limitation", it is of particular interest in mammalian herbivores that consume the foliage of trees and shrubs, because they must metabolize the plant secondary metabolites (PSMs) present to avoid intoxication while still accessing nutrients and water (Dearing 2013). These metabolic processes generate heat, that add to the overall heat load, and at warm ambient temperatures, contribute to the thermoregulatory challenge. For example, when sheep were intravenously infused with the phenolic PSM orcinol, they experienced a 5% increase in MR measured by indirect calorimetry (Iason & Murray 1996). Likewise, when meadow voles, had the phenolic PSM gallic acid incorporated into their diet, they experienced an increase in MR of 14 – 23% (Samson, Thomas & Bergeron 1988). There is a growing body of evidence that warm temperatures reduce the rate at which herbivores can detoxify and hence consume potentially toxic PSMs as a result of this contribution to heat load (Kurnath & Dearing 2013; Connors, Malenke & Dearing 2017).

The marsupial-*Eucalypt* system is one example where a guild of mammalian herbivores consumes potentially toxic plants as a major part of the diet and which has been well studied in both the laboratory and the field. A key determinant of feeding by marsupial folivores is the different classes and concentrations of PSMs contained in *Eucalypt* leaves. For example, koalas eat less of leaves containing high concentrations of formylated phloroglucinol compounds (FPCs) (Moore *et al.* 2005). Likewise, greater

gliders select a diet that minimizes FPC intake, while also attempting to maximize nutrient intake (Jensen, Wallis & Foley 2015). Brushtail possums also ingest less food when it contains higher concentrations of the FPC compound jensenone, so that they do not exceed a threshold level of ingestion (Stapley *et al.* 2000). This level is presumably determined by the rate at which the PSM can be metabolized (Marsh, Wallis & Foley 2005), a physiological process that has traditionally been considered independent of abiotic factors such as ambient temperature, and rather determined by phylogeny and induction of enzymes through past experience. However, the effect of ambient temperature on the nutritional ecology of these species has not been considered.

Eucalyptus is a large genus of some 800 species (Brooker 2000) and earlier work has shown that different species of folivores prefer different groups of eucalypts. This is true of all marsupial folivores that rely on eucalypt foliage and the influence of PSMs on the feeding ecology of koalas (*Phascolarctos cinereus*), greater gliders (*Petauroides volans*), common brushtail (*Trichosurus vulpecula*) and common ringtail possums (*Pseudocheirus peregrinus*) have been well studied (e.g. Foley & Hume 1987; Marsh *et al.* 2003; DeGabriel *et al.* 2010). Both common brushtail possums and common ringtail possums are amenable to captive feeding studies making them ideal candidate study species. Furthermore, brushtail possums tend to eat leaves from trees in the sub-genus *Symphyomyrtus* whereas ringtail possums favour leaves from the sub genus *Eucalyptus* (note that the older name *Monocalyptus* will be used to avoid confusion with the genus name). A key determinant of this differential food selection, is the classes of PSMs contained in each group of trees and the tolerance of each possum species to those PSMs. Unsubstituted B-ring flavanones (UBFs) are one such class of compounds that are present in *Monocalyptus* and absent from *Symphyomyrtus* species, and differentiates feeding by these two sympatric possum species. Brushtail possums are deterred from feeding by the presence of the UBFs, pinocembrin and flavanone, but not deterred by the structurally related non-UBF compounds chrysin (flavone analogue of pinocembrin) or naringenin (flavanone with B-ring substitution) (Marsh *et al.* 2015). In addition, brushtail possums are more generalist herbivores, while ringtail possums are more specialized. In previous studies using woodrats (Genus *Neotoma*), specialists and generalists were found to have different responses in terms of the energy cost of

ingesting novel or non-novel PSMs (Sorensen, McLister & Dearing 2005). Furthermore, given the effects of size on overall metabolism and thermoregulation, by studying these two species in captivity, some variation can be captured.

Since intake of PSMs is determined by the capacity of the animal to absorb, distribute, metabolize and excrete (ADME) the compound, abiotic effects on the metabolism of PSMs could alter food intake patterns. Understanding how changes to ambient temperature may alter one of the most basic and necessary functions of herbivores, feeding, is imperative in predicting changes to ecosystems and planning for future conservation efforts. However, while it is well established that PSMs are an important determinant of food intake, so far attempts to predict the impacts of future climate change, have neglected to include PSMs as a key determinant of animal responses to increased temperatures. Physiologically based species distribution models for example, attempt to integrate physiological responses to environmental features within the current distribution of animals to determine mechanistically what drives broader patterns. These patterns can then be used to predict how animal populations will respond to future conditions. Importantly though, the quality of these predictions is determined by adequate identification and inclusion of relevant factors, and given the importance of PSMs for marsupial folivores, their exclusion is curious. For instance, greater gliders are specialist eucalypt folivores, and PSMs are a key determinant of feeding in this species, however in creating a physiologically based mechanistic niche model, PSMs were not included while nutrients were. Further, consideration of the impact of temperature on feeding rates is often absent, or hidden within estimates of metabolic rate. If temperature influences the tolerance of herbivores to PSMs, then we should seek to better understand this dynamic relationship so that it may be included in models, and conservation efforts in the future.

Thesis outline

In this thesis, I aimed to address the question, how does ambient temperature influence feeding by marsupial folivores? I focused on how ambient temperature and the presence of PSMs in the diet of marsupial folivores influences temperature dependent toxicity, macronutrient balancing, and mitochondrial uncoupling, and in doing so

tackled this question from the cell level to free ranging animals. To do so, I present the following six chapters, followed by an overall thesis synthesis:

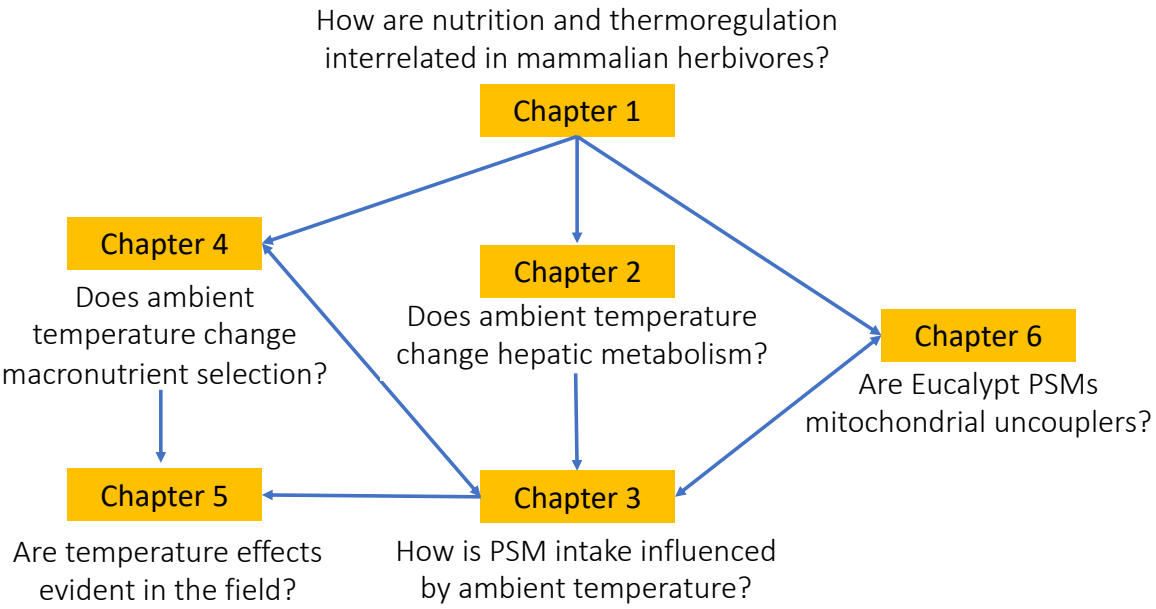

Figure 1: *Thesis structure including main questions addressed in each chapter*

Chapter 1 will review the current literature examining the physiological interactions between diet and changes in ambient temperature in mammalian herbivores in general. It highlights how consuming plants may make these animals particularly susceptible to the negative influences of increased ambient temperature. It comprises a discussion of the thermal relations of mammals, obligatory diet-induced thermogenesis, facultative diet-induced thermogenesis from unbalanced diets, temperature dependent toxicity, as well as direct effects of plant secondary metabolites on thermoregulation in animals. In addition, predictions are made about how these diet-temperature interactions will affect herbivore populations *in situ* in the environment under future climate change. This chapter has been published in Biological Reviews of the Cambridge Philosophical Society (Beale *et al.* 2018).

In Chapter 2 I investigated the concept of temperature-dependent toxicity. I tested whether the hepatic detoxification capacity of brushtail and ringtail possums was altered by exposure to different ambient temperatures. I also considered if the length

of exposure to different temperatures modulated the effect of ambient temperature by exposing both species of possums to a single ambient temperature for either one week or one day. To measure the hepatic detoxification capacity, I use the anesthetic agent Alfaxan in a sleep time assay as a proxy for PSM metabolism. This chapter is formatted for submission to Functional Ecology.

In Chapter 3, I tested whether the changes to hepatic function observed in Chapter 2 were reflected in changes of food intake by both brushtail and ringtail possums. This chapter includes four captive animal feeding experiments, short and long temperature exposure experiments for each species. Ringtail possums were fed a leaf-based diet from trees of known chemical composition, while brushtail possums were fed formulated diets with or without a PSM. To determine the temperatures to be used for experiments throughout, I measured relationships between ambient temperature, metabolic rate and respiration in ringtail possums, to estimate their thermoneutral zone. These data are included in this chapter however it is also of relevance to Chapter 2 and Chapter 4. This chapter is formatted for submission to the Journal of Comparative Physiology B.

Chapter 4 investigates the interplay between temperature, macronutrient selection, and PSM intake in brushtail possums using captive feeding experiments. Availability of protein is important for determining herbivore abundance and distribution and in influencing the reproductive success of common brushtail possums at least. Furthermore, PSM ingestion appears to induce a significant elevation in protein turnover so that ingesting more protein also allows ingestion of more PSMs. However, of the macronutrients, protein has a higher heat increment (the energy required for digestive and absorptive processes) than either carbohydrates or fats, making protein-rich food, more thermogenic. I used nutritional geometry to interpret the results showing ambient temperature can influence macronutrient selection, and how this is altered by the presence of PSMs.

In Chapter 5, I used an existing dataset on feeding rates of koalas which was originally collected by Dr Karen Marsh, one of my supervisory panel. These data were collected from koalas equipped with radio location collars that also contained a microphone that recorded all feeding sounds. This allowed meal lengths to be estimated in many trees of

known chemical composition. I extended the original analyses by relating the nutritional and chemical quality of these leaves, with ambient temperature both at the times of the meals and across days to look for field-based evidence of the temperature effects on nutrition found in my previous chapters. This is a starting point for developing methods to translate laboratory-based studies of temperature effects on feeding to field situations.

A variety of different methods have been used in the past to test a range of PSMs from *Eucalyptus* for activity as uncouplers, making results difficult to compare. In Chapter 6 I investigate using a single *in vitro* protocol, whether a range of PSMs may act as mitochondrial uncouplers. I conducted mitochondrial stress tests on rat cells treated with a range of PSMs of relevance to feeding behaviors of a variety of marsupial folivores.

I use the final synthesis chapter to bring the data together and to suggest future directions for investigation. For example, while I have focused on hepatic metabolism, there are other parts of ADME that may be affected by ambient temperature and I discuss these as other potential directions for investigation. Body heat could be measured using calorimetry to determine the exact heat associated with different PSMs. In depth pharmacokinetic studies could be used to more precisely measure the path PSMs take through the body of herbivores and how this changes with heat exposure. Or perhaps larger scale field studies could be used to generalize the results to other temperature regimes or to other plant-herbivore systems. I then relate my results to other herbivore systems to place them in a boarder context. There are many possible avenues for future research and expanding the question to “how does ambient temperature influence feeding by herbivores” certainly warrants further thought, particularly in the face of climate change.

References

Beale, P.K., Marsh, K.J., Foley, W.J. & Moore, B.D. (2018) A hot lunch for herbivores: physiological effects of elevated temperatures on mammalian feeding ecology. *Biological Reviews of the Cambridge Philosophical Society,* **93,** 674-692.

Brooker, M.I.H. (2000) A new classification of the genus *Eucalyptus* (Myrtaceae). *Australian systematic botany,* **13,** 79-148.

Connors, P.K., Malenke, J.R. & Dearing, M.D. (2017) Ambient temperature-mediated changes in hepatic gene expression of a mammalian herbivore (*Neotoma lepida*). *Molecular Ecology,* **26,** 4322-4338.

Dearing, M.D. (2013) Temperature-dependent toxicity in mammals with implications for herbivores: a review. *Journal of Comparative Physiology B,* **183,** 43-50.

DeGabriel, J.L., Moore, B.D., Marsh, K.J. & Foley, W.J. (2010) The effect of plant secondary metabolites on the interplay between the internal and external environments of marsupial folivores. *Chemoecology,* **20,** 97-108.

Foley, W.J. & Hume, I.D. (1987) Nitrogen Requirements and Urea Metabolism in Two Arboreal Marsupials, the Greater Glider (*Petauroides volans*) and the Brushtail Possum (*Trichosurus vulpecula*), Fed Eucalyptus Foliage. *Physiological Zoology,* **60,** 241-250.

Hoegh-Guldberg, O., Jacob, D., Taylor, M., Bindi, M., Brown, S., Camilloni, I., Diedhiou, A., Djalante, R., Ebi, K., Engelbrecht, F., Guiot, J., Hijioka, Y., Mehrotra, S., Payne, A., Seneviratne, S., Thomas, A., Warren, R. & Zhou, G. (2018) Impacts of 1.5ºC global warming on natural and human systems. In: Global warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty. (ed. P.Z. V. Masson-Delmotte, H. O. Pörtner, D. Roberts, J. Skea, P.R. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J. B. R. Matthews, Y. Chen, X.

Zhou, M. I. Gomis, E. Lonnoy, T. Maycock, M. Tignor, T. Waterfield). The Intergovernmental Panel on Climate Change.

Iason, G.R. & Murray, A.H. (1996) The energy costs of ingestion of naturally occurring nontannin plant phenolics by sheep. *Physiological Zoology,* **69,** 532-546.

Jensen, L.M., Wallis, I.R. & Foley, W.J. (2015) The relative concentrations of nutrients and toxins dictate feeding by a vertebrate browser, the greater glider *petauroides volans*. *PLoS ONE,* **10,** e0121584-e0121584.

Kurnath, P. & Dearing, M.D. (2013) Warmer ambient temperatures depress liver function in a mammalian herbivore. *Biology Letters,* **9,** 20130562.

Marsh, K.J., Foley, W.J., Cowling, A. & Wallis, I.R. (2003) Differential susceptibility to Eucalyptus secondary compounds explains feeding by the common ringtail (*Pseudocheirus peregrinus*) and common brushtail possum (*Trichosurus vulpecula*). *Journal of Comparative Physiology B,* **173,** 69-78.

Marsh, K.J., Wallis, I.R. & Foley, W.J. (2005) Detoxification rates constrain feeding in common brushtail possums (*Trichosurus vulpecula*). *Ecology,* **86,** 2946-2954.

Marsh, K.J., Yin, B., Singh, I.P., Saraf, I., Choudhary, A., Au, J., Tucker, D.J. & Foley, W.J. (2015) From leaf metabolome to in vivo testing: identifying antifeedant compounds for ecological studies of marsupial diets. *Journal of Chemical Ecology,* **41,** 513-519.

Moore, B.D., Foley, W.J., Wallis, I.R., Cowling, A. & Handasyde, K.A. (2005) *Eucalyptus* foliar chemistry explains selective feeding by koalas. *Biology Letters,* **1,** 64-67.

Samson, C., Thomas, D.W. & Bergeron, J.-M. (1988) Metabolic costs associated with the ingestion of plant phenolics by *Microtus pennsylvanicus*. *Journal of Mammalogy,* **69,** 512-515.

Sorensen, J.S., McLister, J.D. & Dearing, M.D. (2005) Plant secondary metabolites compromise the energy budgets of specialist and generalist mammalian herbivores. *Ecology,* **86,** 125-139.

Speakman, J.R. & Król, E. (2010) Maximal heat dissipation capacity and hyperthermia risk: neglected key factors in the ecology of endotherms. *Journal of Animal Ecology,* **79,** 726-746.

Stapley, J., Foley, W.J., Cunningham, R. & Eschler, B. (2000) How well can common brushtail possums regulate their intake of *Eucalyptus* toxins? *Journal of Comparative Physiology B,* **170,** 211-218.

Chapter 1

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A hot lunch for herbivores: physiological effects of elevated temperatures on mammalian feeding ecology

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ABSTRACT

Mammals maintain specific body temperatures (T_b) across a broad range of ambient temperatures. The energy required for thermoregulation ultimately comes from the diet, and so what animals eat is inextricably linked to thermoregulation. Endothermic herbivores must balance energy requirements and expenditure with complicated thermoregulatory challenges from changing thermal, nutritional and toxicological environments. In this review we provide evidence that plant-based diets can influence thermoregulation beyond the control of herbivores, and that this can render them susceptible to heat stress. Notably, herbivorous diets often require specialised digestive systems, are imbalanced, and contain plant secondary metabolites (PSMs). PSMs in particular are able to interfere with the physiological processes responsible for thermoregulation, for example by uncoupling mitochondrial oxidative phosphorylation, binding to thermoreceptors, or because the pathways required to detoxify PSMs are thermogenic. It is likely, therefore, that increased ambient temperatures due to climate change may have greater and more-specific impacts on herbivores than on other mammals, and that managing internal and external heat loads under these conditions could drive changes in feeding ecology.

Key words: thermoregulation, metabolism, diet, herbivory, thermogenesis, uncoupling, plant secondary metabolite, temperature-dependent toxicity, climate change, heat dissipation limitation.

CONTENTS

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I. INTRODUCTION

In endotherms, body temperature (T_b) is one of the most carefully defended physiological traits. Through mechanisms that control the generation, conservation and dissipation of heat, endotherms can maintain a relatively constant T_b across a broad range of ambient temperatures (Rezende & Bacigalupe, 2015). Thus, thermogenesis and ambient temperature are inextricably and fundamentally linked. Differences in cellular processes, particularly those that alter membrane permeability, contribute to the T_b of different groups of endotherms. In fact, leakage of ions across cell and mitochondrial membranes throughout the entire body, and the active pumping of these ions against countervailing concentration gradients, is what makes endotherms energy-expensive, heat-generating organisms (Hulbert & Else, 1990; Else, Turner & Hulbert, 2004).

In a recent phylogenetic analysis of T_b in endotherms, Clarke & O'Connor (2014) found a significant relationship between T_b and diet in mammals. Although T_b can be subject to seasonal variation and photoperiod control (e.g. Turbill *et al.*, 2011), herbivory is associated with a higher mean T_b than carnivory (Clarke & O'Connor, 2014). In addition, mammalian herbivores that feed on fibre-rich diets tend to have higher mean T_b than frugivores, which have an intermediate T_b, and flower and nectar feeders which have the lowest mean T_b (Clarke & O'Connor, 2014). Higher T_b has also been linked to herbivory in other taxa, e.g. birds (Clarke & O'Connor, 2014) and reptiles (Pough, 1973; Espinoza, Wiens & Tracy, 2004).

The digestion and metabolism of macronutrients and plant secondary metabolites (PSMs) are exothermic processes, and some PSMs also have the capacity to interfere with thermoregulation. These characteristics of herbivorous diets may present challenges for animals experiencing high ambient temperatures, because they have less opportunity to dissipate any heat produced. Under most climate-change scenarios, extreme events, including droughts and heat waves, are expected to increase in both frequency and severity, and temperature minima and maxima are expected to rise (Parry *et al.*, 2007). While less predictable, there may also be changes in relative humidity (Zurbenko & Luo, 2015). Understanding the links between thermoregulation, diet, and ambient temperature will be invaluable in predicting animal responses to climate change.

In this review, we examine how the macronutrient and PSM composition of diets can influence thermoregulatory processes. Much of the work in this area comes from pharmaceutical and domestic animal studies, with a lack of ecologically relevant data for wild endotherms. Nevertheless, we show that temperature-dependent toxicity of PSMs and limits to heat dissipation are likely to have a strong influence on mammalian herbivore–plant interactions, particularly at high ambient temperatures.

II. BODY TEMPERATURE REGULATION IN MAMMALS

(1) The thermoneutral zone

The range of ambient temperatures at which heat generated by resting metabolism is equal to net heat lost into the environment, resulting in maintenance of Tb, is known as the thermoneutral zone (TNZ) (Scholander, 1955). Within the TNZ, behavioural, postural and vasomotor adjustments are sufficient for matching heat generation with heat loss. Below the lower critical temperature (LCT) of the TNZ, heat lost to the environment is greater than that generated through basal metabolism, so the animal initiates both a reduction in heat loss (e.g. peripheral vasoconstriction and piloerection) and an increase in heat generation (Gordon, Johnstone & Aydin, 2014). Heat may be generated by shivering thermogenesis, non-shivering thermogenesis, or activity (Rezende & Bacigalupe, 2015). Shivering thermogenesis is the generation of heat from involuntary muscular contractions and hydrolysis of ATP without any productive movement (Cannon & Nedergaard, 2004). By contrast, non-shivering thermogenesis requires an increase in metabolic rate through uncoupling of oxidative phosphorylation in mitochondria, particularly in tissues of high oxidative capacity (Cannon & Nedergaard, 2004).

At ambient temperatures above the upper critical temperature (UCT), metabolic rate also increases, but this is due to active cooling mechanisms, such as sweating or panting (Rezende & Bacigalupe, 2015). At high ambient temperatures, and in humid conditions, the physiological capacity to dissipate additional heat is limited. This means that there is a smaller margin before the limits of survival are reached at high ambient temperatures compared to cold (Cannon & Nedergaard, 2011). High levels of radiant heat also contribute to external heat load, and high humidity and low convection make evaporative heat loss more difficult. For this reason, indices used for quantifying external heat load often include some combination of temperature, humidity, radiation, and convection, and responses to high ambient temperature can be modified by variation in these other external parameters (Gaughan *et al.*, 2008). Endotherms must carefully regulate processes that could

lead to an increase in body heat production when nearing their UCT, or risk hyperthermia.

(2) Limitations to heat dissipation

The idea that physiological processes may be limited by an animal's capacity to dissipate sufficient heat so as to maintain T_b was recently termed the "heat dissipation limitation" (HDL) hypothesis (Speakman & Król, 2010). Evidence for HDL comes from studies of lactating mice that did not increase food intake or milk production despite increased litter sizes (Hammond & Diamond, 1992; Johnson, Thomson & Speakman, 2001), concurrent pregnancy (Johnson & Thomson, 2001), extended lactation (Hammond & Diamond, 1994; Laurien-Kehnen & Trillmich, 2003) or forced exercise (Perrigo, 1987). However, interventions that enhanced heat loss, such as housing animals at a low ambient temperature or reducing insulation (shaving of dorsal fur), resulted in increased food intake, greater milk production, and heavier offspring (Król, Murphy & Speakman, 2007; Speakman & Król, 2010). Thus, the ability to dissipate heat produced by exothermic processes can ultimately limit energy intake and utilisation for an endotherm.

The HDL can be applied to any process that produces heat as a by-product, resulting in a trade-off with thermoregulation at high ambient temperatures. Although reproduction and physical activity are conspicuous examples of exothermic processes, the digestion of food also produces heat and thus may be constrained by thermoregulation. Reduced food intake and productivity have long been recognised as consequences of heat stress in domestic animals (Hammond & Diamond, 1994; Konarzewski & Diamond, 1994; Speakman, 2000; Bacigalupe & Bozinovic, 2002), but few studies have investigated the relationship between diet selection and ambient temperature in wild endotherms (although see Kurnath & Dearing, 2013; Kurnath, Merz & Dearing, 2016).

III. FOOD INTAKE AT HIGH AMBIENT TEMPERATURES

Much of what we know about the impact of ambient temperature on feeding rates comes from domestic animals. Increased food intake below the TNZ can be explained by increased metabolic rate due to the demands of thermogenesis. Although metabolic rate is also increased by thermoregulatory demands above the TNZ, food intake is reduced, often dramatically (e.g. up to 45%; Renaudeau, Quiniou & Dubois, 2002). This has been observed repeatedly for domestic cattle (Bernabucci *et al.*, 1999; Ominski *et al.*, 2002), pigs (Close, Mount & Start, 1971; Prunier, de Bragança & Le Dividich, 1997; Renaudeau *et al.*, 2002) and poultry (Dale & Fuller, 1979; Baziz *et al.*, 1996). In many of these studies, heat stress also alters body composition, resulting in the accumulation of fat rather than lean body mass (Rhoads *et al.*, 2013). This is because chronic heat stress reduces protein synthesis and increases protein catabolism (Bianca, 1965; Hall *et al.*, 1980; Marder *et al.*, 1990; Wheelock *et al.*, 2010). Increased protein catabolism likely results in increased gluconeogenesis (Collins, Mitros & Skibba, 1980; Baumgard & Rhoads, 2012). However, after chronic heat exposure, blood glucose is often reduced regardless of increased intestinal absorptive capacity, hepatic output and renal resorption, indicating negative energy balance (Baumgard & Rhoads, 2012; Belhadj Slimen *et al.*, 2016). Therefore, chronic heat exposure shifts the use of energy substrates away from fatty acid oxidation towards glucose use (Baumgard & Rhoads, 2012).

Changes in maintenance requirements in response to ambient temperature are clearly not the sole drivers of food intake. Rather, there are significant interactions between diet, energy balance, thermoregulation and ambient temperature. In both wild and domestic herbivores, there can be additional seasonal variation in fat deposition triggered by photoperiod and subject to hormonal regulation (Nagy, Gower & Stetson, 1995; Faulconnier *et al.*, 2001; Lincoln *et al.*, 2001). However, the overall decrease in food intake seen at high ambient temperatures in domestic animals is thought to be driven by the interaction between diet-induced thermogenesis (DIT) and the need to balance heat production and dissipation to maintain T_b (Herd, Oddy & Richardson, 2004; Renaudeau *et al.*, 2012).

IV. DIET-INDUCED THERMOGENESIS AS A SOURCE OF HEAT

Diet-induced thermogenesis (DIT) is the increase in metabolic heat production following the ingestion of food. It is worth noting that terminology associated with this topic is not uniform, and DIT has been used interchangeably with 'specific dynamic action' (SDA), the 'thermic effect' of food (TE), and the 'heat increment' (HI) or 'heat increment of feeding' (HIF).

DIT has both an obligatory (ObT) and a facultative component (FcT). ObT is the heat that is produced by the digestion, absorption and assimilation of nutrients, while FcT is heat produced by metabolic uncoupling for the regulation of energy balance. FcT is also called 'adaptive thermogenesis' and 'regulated thermogenesis'. Eutherians and marsupials may differ in the importance and control of FcT *versus* ObT (Dawson & Olson, 1988).

Many studies investigate DIT as a whole, rather than distinguishing between FcT and ObT components, and some studies have investigated FcT or ObT but refer to their results as DIT (e.g. Feldmann *et al.*, 2009). There are also both obligatory and facultative components to overall thermoregulation outside of the DIT umbrella, but they will not be discussed in detail here.

(1) Obligatory thermogenesis

Larger meals cause greater ObT (and therefore DIT), higher peak metabolism and a more prolonged effect compared to

smaller meals (see Secor, 2009 for a comprehensive review). Some authors report a linear relationship between meal size (wet mass of the meal or wet mass of the meal as a percentage of body mass) and DIT (Ross *et al.*, 1992; Secor & Diamond, 1997; Fu, Xie & Cao, 2005), while others have observed a plateau in DIT as meal size becomes very large (Jobling & Davies, 1980; Secor & Boehm, 2006; Secor, 2009). This plateau may result from limits to the oxidative capacity of the tissues, or alternatively, limits to the digestion and transport of nutrients in the gut (Jobling & Davies, 1980; Secor, 2009).

Endotherms ingesting foods which are cooler than their own T_b generate extra heat to warm the food (Berteaux, 2000; Secor, 2009). This energy cost is included in DIT, and will be influenced by the mass of food ingested and its temperature (Berteaux, 2000; Secor, 2009). Unsurprisingly, larger and cooler meals require more energy to warm to T_b and are therefore accompanied by larger increases in DIT (Berteaux, 2000; Secor, 2009).

Environmental temperature can also influence the measured DIT, such that at low ambient temperatures, the same meal will appear to have a smaller DIT than at high ambient temperatures. This is due to compensation – heat produced by digestion substitutes for specific thermogenesis that would otherwise be required to maintain T_b under cold conditions (Chappell, Bachman & Hammond, 1997). In this way DIT is largely hidden by total metabolic rate adjustments at cool temperatures, and may explain why DIT is not always observed (Campbell, McIntyre & MacArthur, 2000; Rosen & Trites, 2003). Compensation may be more useful for animals residing in colder habitats (Campbell *et al.*, 2000), or potentially, animals experiencing significant temperature variability, as metabolic adjustments provide a faster response to cold than alternatives such as producing extra insulation.

Another factor that influences ObT is the nutritional composition of the meal. This is because (i) different macronutrients make different contributions to ObT, and (ii) the specific amount of heat released during metabolism or detoxification of a PSM depends on the change in enthalpy during the chemical processes that contribute to Phase 1 (oxidation, reduction, hydrolysis) and Phase 2 (conjugation) metabolism of that PSM (Dearing, Foley & McLean, 2005).

In humans, 0–3% of the energy available in fat, 5–10% of the energy available in carbohydrates, and 20–30% of the energy available in protein is expended by ObT. In an energy-balanced state this should account for approximately 10% of total daily energy expenditure (Acheson, 1990; Westerterp, 2004). The relative contributions of these macronutrients to ObT are consistent across taxa (Secor, 2009). In some herbivores, however, heat produced by digestion of dietary fibre surpasses the contribution of other macronutrients to DIT (Secor, 2009). The guts of many herbivores are specialised to process large amounts of otherwise indigestible fibre (largely cellulose and hemicellulose) by bacterial fermentation (Stevens & Hume, 1998). For example, in large ruminant herbivores, 60–70% of energy absorbed from the diet can be derived from fibre digestion (Egan, 1989; Devendra & Leng, 2011). The main end products of bacterial fermentation include the short-chain fatty acids (SCFAs), acetate, propionate and butyrate, and microbial protein (Stevens & Hume, 1998). High-fibre diets favour the production of acetate over propionate. Inefficient ATP production using acetate as a substrate is now thought, in part, to cause the high DIT measured in ruminants (Cho *et al.*, 2014). As testament to the thermogenic effects of acetate, this substrate is preferentially oxidised when an animal is cold-challenged, leaving larger amounts of amino acids relative to glucose available for growth and reproduction than would be available at thermoneutrality (Baumgard & Rhoads, 2012). When animals are heat stressed, diets relatively low in dietary fibre, and more energy dense (e.g. containing more fat), are recommended to ameliorate depressed food intake (West, 1999).

Plant secondary metabolites are ubiquitous in the diets of browsing herbivores and frugivores. Although some have a demonstrated role as antifeedants, most of their effects are uncharacterised (Dearing *et al.*, 2005; Forbey & Foley, 2009). The metabolic activities of the liver, including the detoxification of PSMs, contribute significantly to heat generation in the body (Berry *et al.*, 1985; Wang *et al.*, 2010). For example, protein turnover, one of the major components of metabolic heat production is significantly increased during detoxification (Au *et al.*, 2013). There is a growing body of evidence from pharmaceutical (Keplinger, Lanier & Deichmann, 1959; Weihe, 1973; Gordon *et al.*, 1988*a*), agricultural (Aldrich *et al.*, 1993; Settivari *et al.*, 2008, 2009), and wild herbivore (Chatelain, Halpin & Rowe, 2013; Kurnath & Dearing, 2013; Kurnath *et al.*, 2016) studies supporting the idea that ambient temperature can have dramatic implications for xenobiotic toxicity (Dearing, 2013). This is termed 'temperature-dependent toxicity' (TDT).

(2) PSM metabolism and temperature-dependent toxicity

The earliest evidence for TDT came from laboratory rodents, in which the lethal dose of a variety of plant-derived drugs was reduced at higher ambient temperatures (Keplinger *et al.*, 1959). Later studies revealed that acclimation to warmer temperatures may decrease hepatic gene expression of some enzymes involved in xenobiotic metabolism, and may also decrease hepatic enzyme activity (Kaplanski & Ben-Zvi, 1980; Settivari *et al.*, 2009). In more ecologically relevant examples, TDT has been observed in several species of herbivorous woodrats (*Neotoma* spp.) (Kurnath & Dearing, 2013; Kurnath *et al.*, 2016) and in vertebrate pests during poisoning campaigns (Oliver & King, 1983).

Hypnotic state (sleeping time) assays using hexobarbital showed that liver function in woodrats acclimated to high ambient temperatures was depressed, and thus clearance of the hexobarbital took longer than at low temperatures (Kurnath & Dearing, 2013). Woodrats also demonstrate temperature-dependent changes in diet selection to reduce intake of PSMs at high ambient temperatures (Kurnath *et al.*, 2016). Studies suggest that reduced detoxification capacity in the liver at high ambient temperatures could be due to

one or more of the following: a decrease in expression of genes coding for enzymes involved in detoxification; reduced activity of critical enzymatic reactions; or lower liver mass (Dearing, 2013; Gordon *et al.*, 2014). As ambient temperature increases, the physiological capacity of herbivores to deal with PSMs therefore decreases. This can be true even within the TNZ (Kurnath *et al.*, 2016).

The fundamental reason for down-regulation of detoxification systems by endotherms at high ambient temperatures is likely to be limitations to heat dissipation. Heat produced by detoxification of PSMs must be able to be dissipated, or the animal risks hyperthermia. Any thermogenic action of PSMs can be considered to alter the ObT component of DIT, as the amount of heat produced from the animals' perspective can only be changed *via* the amount of food taken in. Thus, in accordance with the HDL hypothesis, it is likely that animals would defend T_b by reducing DIT through eating less of diets containing PSMs at high ambient temperatures. As suggested by Kurnath *et al.* (2016), the interaction between ambient temperature and liver function could have critical implications for mammalian herbivores that must balance PSM detoxification with thermoregulation, particularly in a warming environment.

(3) Facultative thermogenesis

The FcT component of DIT is a means for maintenance of energy homeostasis. Early researchers noted that energy intake could exceed energy allocation to growth and maintenance, suggesting that some of the metabolisable energy from the diet was converted to heat and dissipated (Miller & Payne, 1962). Later, Rothwell & Stock (1979) showed that rats fed cafeteria diets had higher non-shivering thermogenic capacity than those fed formulated diets. Stock (1999) points to a scarcity of obesity-prone phenotypes as evidence for the adaptive advantage of energetically wasteful thermogenic mechanisms, i.e. FcT. However, he also points out that these mechanisms must be regulated so that they can be switched off either when energy availability is limited, or energy demand is increased, such as during pregnancy or lactation.

Whereas all tissues contribute to thermogenesis in varying amounts, the primary tissue responsible for FcT in eutherian mammals is considered to be brown adipose tissue (BAT) (although see Kozak, 2010). The thermogenic capacity of BAT is attributable almost entirely to the actions of uncoupling protein 1 (UCP1) (Cannon & Nedergaard, 2004). UCP1 is an anion-carrier protein on the inner mitochondrial membrane, which allows protons to pass between the inter-membrane space and the mitochondrial matrix (Fig. 1B). This protonophoric activity results in dissipation of the electrochemical gradient, which provides the proton-motive force required by ATP synthase to convert ADP to ATP (Fig. 1A). As a result, more glucose and oxygen are consumed by the electron transport chain to generate the gradient and form the same amount of ATP (Lowell & Spiegelman, 2000; Krauss, Zhang & Lowell, 2005). Uncoupling refers to this mismatch between oxygen consumption (cellular respiration) and ATP synthesis. The chemical energy consumed through this 'futile cycling' of ions is dissipated from mitochondria as heat (Silva & Rabelo, 1997; Cannon & Nedergaard, 2004).

FcT is regulated centrally by the sympathetic nervous system, and several key hormones are involved in the process (Cannon & Nedergaard, 2004; Fig. 2). FcT is influenced by diet, environmental temperature, and likely, by many genes. For example, FcT is initiated in response to chronic over-eating (Fig. 2). Like any thermogenesis, FcT will be restricted by the ability to dissipate the heat produced. Consequently, FcT may be less useful as an energy-balancing mechanism at high ambient temperatures. Since the action of UCP1 is up-regulated in both cold acclimation and in times of excess energy intake, animals that routinely have a total energy intake that exceeds their requirements are also effectively cold-acclimated, having up-regulated some of the same physiological mechanisms as would occur in response to chronic cold exposure.

The lack of BAT and UCP1 in some eutherian mammals (e.g. Berg, Gustafson & Andersson, 2006), its down-regulation in human adults (Rowland, Bal & Periasamy, 2015), and controversy surrounding its presence in marsupials (Jastroch *et al.*, 2008), has led to a search for other thermogenic mechanisms. Futile cycling of Ca^{2+} by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA1) pump in skeletal muscle has been proposed as a complementary method of facultative thermogenesis in mammals (Arruda *et al.*, 2008; Little & Seebacher, 2014; Rowland *et al.*, 2015), particularly larger mammals (Rowland *et al.*, 2015), and is the primary site for FcT in birds (Teulier *et al.*, 2010). This ion pump is abundant in skeletal muscle (Vangheluwe *et al.*, 2005; de Jonge *et al.*, 2006; Babu *et al.*, 2007; Rowland *et al.*, 2015), its activity can be regulated by cellular conditions (de Meis, 2002; de Meis, Arruda & Carvalho, 2005; Arruda *et al.*, 2008; Kjelstrup *et al.*, 2008; Mahmmoud, 2008), and its action can be uncoupled from ATP hydrolysis by sarcolipin (SLN) (Smith *et al.*, 2002; Mall *et al.*, 2006; Bal *et al.*, 2012). SLN knockout mice develop hypothermia at 4°C (Bal *et al.*, 2012), and SERCA1 expression is increased in the red muscle of rabbits acclimated to cold ambient temperatures (Arruda *et al.*, 2008). In addition, overexpression of SLN enhances energy expenditure and provides resistance against diet-induced obesity (Bal *et al.*, 2012; Maurya *et al.*, 2015). Mitochondrial creatinine kinases may also play a role in FcT (Müller *et al.*, 2016). The potential role of these skeletal muscle-based thermogenic mechanisms in FcT in marsupials is yet to be confirmed, as is the role of thermogenesis in the liver (Rowland *et al.*, 2015). In fact, our understanding of how these alternative thermogenic mechanisms operate is in its infancy. How they may interact with macronutrients in the diet is an area for future research.

(4) Nutrient balancing using facultative thermogenesis

Wild herbivores are faced with a heterogeneous nutritional landscape of plants with varying concentrations of nutrients

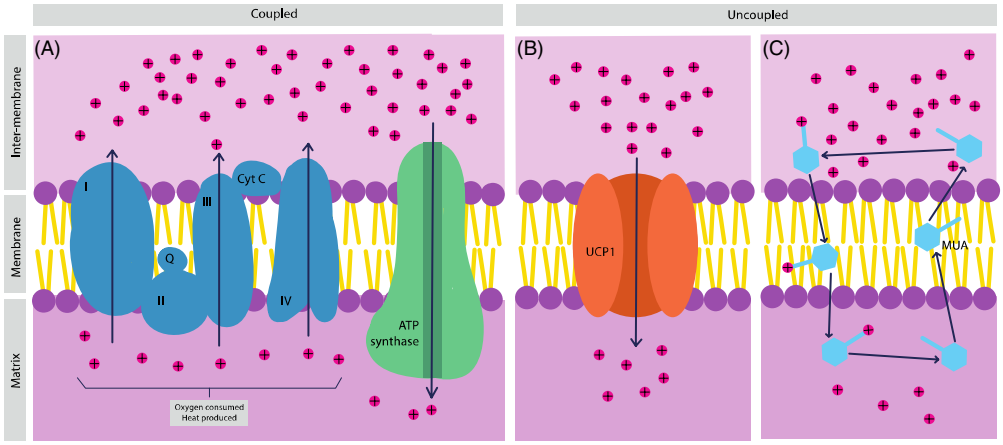

Fig. 1. Mechanisms of mitochondrial uncoupling. (A) 'Coupled' mitochondria. The electron transport chain generates an electrochemical gradient by pumping protons from the mitochondrial matrix through the inner mitochondrial membrane, into the inter-membrane space. This process consumes oxygen and generates heat. The electrochemical gradient is required so that protons move through ATP synthase down the electrochemical gradient, allowing it to phosphorylate ADP into ATP. (B, C) Two ways in which oxygen consumption can be 'uncoupled' from ATP synthesis. (B) Uncoupling protein 1 (UCP1) on the inner mitochondrial membrane. UCP1 acts as a channel through which protons can pass from the inter-membrane space to the mitochondrial matrix. (C) A classical mitochondrial uncoupling agent (MUA) or 'proton shuttle' moving protons *via* 'perpetual disequilibirum'. As a weak acid, the MUA readily associates and dissociates from protons. The neutral species diffuses across the inner mitochondrial membrane into the mitochondrial matrix (down a chemical gradient) and dissociates into an ionised form. The ionised form is driven back into the intermembrane space due to the strong electrical potential across the membrane (down an electrical gradient). A proton is carried across the inner mitochondrial membrane with each iteration of the cycle. The result of both UCP1 in B and the MUA in C is dissipation of the electrochemical gradient required by ATP synthase. This means that, to generate the same amount of ATP, the electron transport chain must work harder, consuming more oxygen and generating more heat in the process.

and PSMs. An animal ingesting a single nutritionally balanced food would only need to eat until its energy and protein requirements were met simultaneously. However, an animal eating foods with insufficient protein, but excess available fats and/or carbohydrate must exceed its total energy requirements in order to meet its protein requirements (Sørensen *et al.*, 2008).

Raubenheimer & Simpson (1997) and Simpson & Raubenheimer (2001) proposed a geometric approach to visualise trade-offs in diet selection amongst nutritional intake targets for different nutrients when feeding on nutritionally unbalanced foods. This approach has been used successfully to show, for example, that wild spider monkeys (*Ateles chamek*) prioritise the acquisition of protein over total energy, and therefore ingest excess energy (Felton *et al.*, 2009).

When excess energy is ingested, fat storage can be useful insurance against times of food limitation. In these 'stochastic shortfalls', individuals that store more fat can survive longer than those that store less (Speakman & Westerterp, 2013). However, as Speakman (2014, p. 92) points out "If the avoidance of starvation was the only criterion that governed the level of fat storage, then one would predict that individuals should always maximise their fat storage levels and the world should be populated only by massively obese animals." In some circumstances, storing large amounts of fat is not possible due to nutritional limitations, but in many situations food supply is not constrained. For example, captive animals given unlimited food, smaller individuals, or non-lactating individuals within a population do not always become obese (Boutin, 1990; Speakman & Król, 2010). In the latter two cases supply is similar to that of their counterparts, even though energy demand is lower. The 'mass-dependent starvation–predation risk trade-off hypothesis' describes the most touted countervailing selection pressure against unchecked fat storage: predation risk (Lima, 1986; Carlsen *et al.*, 1999; Brodin, 2001; MacLeod *et al.*, 2007; Higginson, McNamara & Houston, 2012). Predation risk is increased by loss of speed and agility as fat stores and body mass increase and by the need to spend more time foraging due to higher absolute energy requirements (Speakman, 2014). Having physiological mechanisms to prevent excessive weight gain may be particularly important for individuals who do not hibernate and are forced to over-consume energy in order to meet their requirements for other nutrients (Huang *et al.*, 2013).

Fruit bats (Pteropodidae: Megachiroptera) are argued to be an example that links energy dissipation *via* BAT with a diet that is imbalanced in available energy and protein (Stock, 1999). They typically eat a low-protein (2–5% crude protein) diet (Delorme & Thomas, 1996; Herrera *et al.*, 2002), and have maintenance energy requirements approximately threefold greater (1300 $kJ.kg^{-0.75}.d^{-1}$) than other mammals

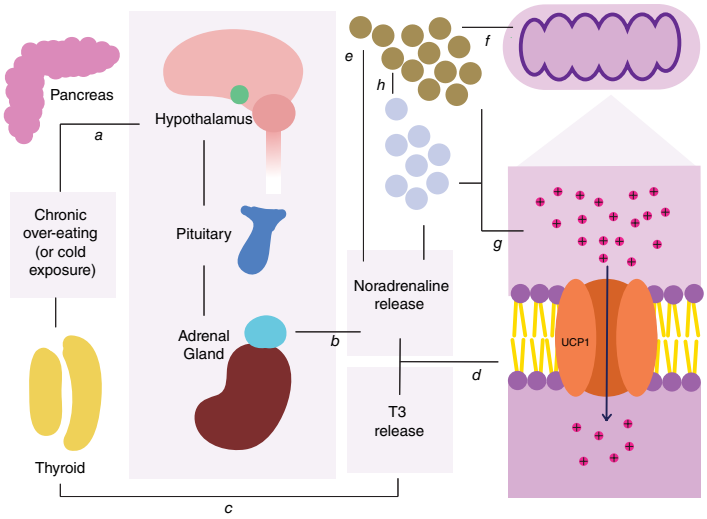

Fig. 2. Facultative thermogenesis in eutherian mammals. Following a meal, (*a*) insulin is released from the pancreas and crosses the blood–brain barrier. Centrally, it has a permissive effect on the hypothalamus to govern thermogenesis *via* the hypothalamic–pituitary–adrenal axis (Silva, 2006; Schwartz *et al.*, 2008). This leads to (*b*) release of noradrenaline (NA) from the adrenal glands (Cannon & Nedergaard, 2004). (*c*) Concurrently triiodothyronine (T3) is released from the thyroid glands and increases the sensitivity of tissues to NA (Silva & Rabelo, 1997). (*d*) During chronic over-eating, the synergistic action of NA and T3 leads to upregulation of uncoupling protein 1 (UCP1) (Silva, 2006), and (*e*) the overall amount of brown adipose tissue (BAT) in the body is increased through hypertrophy and/or hyperplasia of BAT adipocytes. This is paralleled by (*f*) an increase in mitochondrial numbers (Cannon & Nedergaard, 2004). Furthermore, (*g*) free fatty acids are liberated from both BAT and white adipose tissue (WAT) (Cannon & Nedergaard, 2004). These free fatty acids counter inhibition of UCP1 in BAT by purine nucleotides and cause increased glucose transport into the mitochondria, which results in further stimulation of UCP1 [for reviews of BAT in the facultative component of diet-induced thermogenesis (FcT) see Cannon & Nedergaard, 2004 and Lowell & Spiegelman, 2000, and on hormonal control of thermoregulation see Silva, 2006]. More recently, a browning of WAT, to form 'beige fat' has also been recognised (*h*) whereby bipotent pre-adipocyte cells or WAT differentiate into beige adipocytes (Harms & Seale, 2013). These 'beige' adipocytes are similar to BAT, having high thermogenic capacity, and high levels of UCP1 (Harms & Seale, 2013). They clearly play a similar role in thermoregulation and energy balance in laboratory rodents (Ma *et al.*, 1988; Anunciado-Koza *et al.*, 2011; Fromme & Klingenspor, 2011). However, the full extent of this contribution is not yet understood in free-ranging animals attempting to regulate fat storage in a nutritionally challenging landscape (see review by Harms & Seale, 2013).

(420 kJ.kg$^{-0.75}$.d^{-1}) (Delorme & Thomas, 1996). Although they are found mainly in the tropics and sub-tropics, fruit bats have surprisingly well-developed BAT stores (Okon, 1980). Fruit bats appear particularly sensitive to heat stress, as thousands die during heat waves (Welbergen *et al.*, 2008). Interestingly, BAT in fruit bats is most metabolically active during the daytime, rather than at night (Stock, 1999). The HDL hypothesis suggests that an endotherm's ability to expend excess energy through FcT is limited by the rate at which the heat generated can be dissipated into the environment. Total heat load comes from both internal thermogenic processes (e.g. protein synthesis, ion transport, digestion, muscle contraction, lactation), and from external sources (e.g. radiation, conduction, convection). While ambient temperatures are higher during the day, flight and feeding at night also lead to the production of metabolic heat. If the energy that has been ingested in excess of maintenance can be converted to heat, it should be done at a time when the overall heat stored in the body is lowest. In this case, the heat load from flight and feeding may be more than the ambient heat load during the day. It is common for large herbivores to store heat internally (thus sparing water from being used for cooling) until the ambient temperature favours heat dissipation (Gordon *et al.*, 2014; Hetem *et al.*, 2016), highlighting how FcT can be constrained by heat dissipation. Unfortunately, there has been little research to date investigating the use of FcT for energy balance in wild herbivores, or the implications of HDL in this context.

V. THERMOGENIC AND THERMODISRUPTIVE PLANT SECONDARY METABOLITES IN THE DIET

To maintain temperature homeostasis, the body has a system for detecting central and peripheral deviations in temperature, integrating this information, and effecting a

physiological or behavioural change (Bligh, 1966). Any compound that disrupts this process has the potential to alter the animal's ability to respond to thermal challenges. Some PSMs can activate or inhibit both short-term thermoregulatory mechanisms and energy homeostasis. In addition, compounds which act as behaviour modifiers (e.g. chlorpromazine) can prevent behavioural thermoregulation, and have been associated with human drug-related hyperthermia (Weihe, 1973).

(1) Plant secondary metabolites that disrupt thermoception

Several PSMs have been shown to activate TRPV1 (heat) and TRPM8 (cold) thermoreceptors (Table 1). These thermoreceptors are present in different body regions. For example, TRPV1 has been confirmed in skin, gut, bladder, blood vessels, bronchi, tongue and central nervous system, and activation of the same receptor in the gut or the skin may elicit different responses (Premkumar, 2014). Activation of TRPV1 in the skin initiates peripheral vasodilation and heat loss (Varga *et al.*, 2005; Gavva, 2008), while activation of the same receptor in the gut also results in a feeling of satiety (Wang, Miyares & Ahern, 2005).

Capsaicin, the active ingredient in chilli peppers (*Capsicum* spp.), is probably the best studied of these compounds. It binds to the mammalian TRPV1 receptor (Caterina *et al.*, 1997; Premkumar, 2014), causing the ion channel to open, resulting in afferent neurons signalling to the hypothalamus that heat is detected (Premkumar, 2014). The ingestion of capsaicin initially produces vasodilation and sweating (Ahern, 2013), followed by a reflex sympathetic response causing activation of thermogenesis *via* UCP1 in BAT (Ono *et al.*, 2011). Thus, activation of TRPV1 induces both heat-loss and heat-production mechanisms, such that following the initial hypothermic period, a longer-lasting hyperthermic period is experienced (Kobayashi *et al.*, 1998). Ingestion of capsaicin in mice causes increases in thermogenesis 5–20% above basal (Saito, 2015). This has led to the investigation of capsaicin as a potential weight-loss drug (Wang *et al.*, 2005; Ahern, 2013). In addition, capsaicin can cause thermogenesis in skeletal muscle through uncoupling of SERCA1 mediated by TRPV1 binding (Lotteau *et al.*, 2013).

PSMs that bind to TRPM8 cold receptors, such as menthol from mint (*Mentha* spp.) (McKemy *et al.*, 2002; Ma *et al.*, 2012) and 1,8-cineole from *Eucalyptus* (Takaishi *et al.*, 2012), lead to up-regulation of heat-generating mechanisms (Table 1). Menthol can cause increased core temperature, rectal temperature and reduced sweating (Ma *et al.*, 2012). Furthermore, it causes up-regulation of UCP1 in BAT, and hence increased thermogenesis and obesity resistance (Ma *et al.*, 2012).

There are many of these types of thermoreceptors, and many PSMs have been identified as binding to them (see examples in Table 1). When these PSMs are ingested, inappropriate thermoregulatory mechanisms may be initiated in response to misinformation about the thermal environment. These effects can be ongoing, as some potent PSMs, such as resiniferatoxin, found in *Euphorbia resinifera* (Table 1), can cause complete ablation of the nerve terminal through sustained Ca^{2+} influx (Brown *et al.*, 2005; Raisinghani, Pabbidi & Premkumar, 2005; Jeffry *et al.*, 2009; Iadarola & Mannes, 2011; Premkumar, 2014). While there is evidence that activation of these receptors may disrupt thermoregulation, studies of their effects, if any, on wild herbivores ingesting plants that naturally contain these compounds are absent from the literature. It is clear that any compound which disrupts accurate detection of thermal challenge makes thermoregulation more difficult and that these receptors also play a role in energy balance. As a consequence, a need to defend T_b in circumstances of thermal challenge, or perceived thermal challenge, may limit how much of these compounds herbivores can eat.

(2) Plant secondary metabolites that disrupt heat loss mechanisms

In cattle, a condition known as fescue toxicosis, which arises from ingestion of fungal ergot alkaloids on infected pasture, is exacerbated by elevated ambient temperature (Spiers, Evans & Rottinghaus, 2005). This example is often provided as a classic case of TDT, however it is somewhat confounded by the action of the toxin, which interferes with heat loss by limiting vasodilation in the periphery (Aldrich *et al.*, 1993). Similar to this, at high ambient temperatures there have been cases where humans have been hospitalised when taking drugs that act on thermoregulatory mechanisms (Stadnyk & Glezos, 1983; Clark & Lipton, 1984; Vassallo & Delaney, 1989).

The ingestion of tropane alkaloids, such as hyoscyamine, atropine and scopolamine, which are commonly found in the family Solanaceae, frequently results in hyperthermia (Molyneux & Panter, 2009). All three of these compounds act as anticholinergics (Grynkiewicz & Gadzikowska, 2008), meaning that they block the neurotransmitter acetylcholine in nerve synapses of the parasympathetic nervous system (Chang *et al.*, 1999). This causes inhibition of sweating and peripheral vasodilation, resulting in an increase in body temperature through an inability to initiate heat loss (Chang *et al.*, 1999; Grynkiewicz & Gadzikowska, 2008). Sympathomimetic compounds, such as ephedrine, found in *Ephedra sinica*, can inhibit peripheral vasodilation and also increase heat generation (Komissarov, 1964; Ibragic & Sofic, 2015). Amphetamine-related hyperthermia in humans is also due to sympathomimetic action (Asser & Taba, 2015).

(3) Plant secondary metabolites and salts influence water intake and requirements

The renal mechanisms for excretion of PSMs require water (Foley, McLean & Cork, 1995). Ingestion of PSMs in the natural diet of two species of woodrat (*Neotoma albigula* and *Neotoma stephensi*) resulted in increased voluntary water intake and urine output and decreased urine osmolarity in both species (Dearing, Mangione & Karasov, 2001). High

Table 1. Plant secondary metabolites shown to bind to the heat receptor TRPV1 or the cold receptor TRPM8, cause mitochondrial uncoupling through up-regulation of uncoupling protein 1 (UCP1), and/or uncoupling of sarco/endoplasmic reticulum Ca2 + −ATPase 1 (SERCA1) in skeletal muscle. +, agonsim; −, antagonism; NR, not reported; NE, no effect

Compound	TRPV1	TRPM8	UCP1	SERCA1	Plants	References
Capsaicin	+	–	+	+	*Capsicum frutescens* and *C. annum*	Bisogno *et al.* (2001), Caterina *et al.* (1997), Kida *et al.* (2016), Takaishi *et al.* (2016) and Mahmmoud (2008)
Resiniferatoxin (RTX)	++	–	NR	+	*Euphorbia resinifera*	Caterina *et al.* (1997), Koh *et al.* (2016), Mahmmoud & Gaster (2012) and Lotteau *et al.* (2013)
Tinyatoxin (TNX)	++	NR	NR	NR	*Euphorbia poissonii*	Caterina *et al.* (1997)
Eugenol	+	+	NR	NR	Basil (*Ocimum basilicum*), clove (*Syzygium aromaticum*), bay (*Cinnamomum tamalé*), tarragon (*Artemisia dracunculus*)	Bandell *et al.* (2004), Behrendt *et al.* (2004), Yang *et al.* (2003) and Park *et al.* (2009)
Piperine	+	NR	+	NR	Black pepper (*Piper nigrum*)	McNamara, Randall & Gunthorpe *et al.* (2005)
Cannabidiol	+	–	+	NR	Cannabis (*Cannabis sativa*)	Kim *et al.* (2017) Bisogno *et al.* (2001), de Petrocellis *et al.* (2008), Kaneko & Szallasi (2014), Parray & Yun (2016) and Mahmmoud & Gaster (2012)
Camphor	+	+	NR	NR	*Cinnamomum camphora*, rosemary (*Rosarium officals*)	Macpherson *et al.* (2006), Xu, Blair & Clapham *et al.* (2005) and Selescu *et al.* (2013)
Evodiamine	+	–	NE	NR	*Evodia ruticarpa*	Pearce *et al.* (2004) and Wang *et al.* (2008)
Gingerols	+	NR	+	+	Ginger (*Zingiber officinale*), grains of paradise (*Aframomum melegueta*)	Bandell *et al.* (2004), Morera *et al.* (2012), Sugita *et al.* (2013) and Kobayashi, Shoji, & Ohizumi (1987)
Shogaols	+	NE	+	NR	Ginger (*Zingiber officinale*), grains of paradise (*Aframomum melegueta*)	Bandell *et al.* (2004), Morera *et al.* (2012) and Riera *et al.* (2009)
Polygodial	+	NR	NR	NR	Ginger (*Zingiber officinale*),	Bandell *et al.* (2004) and Morera *et al.* (2012)
Thymol	+	–	+	–	Thyme (*Thymus vulgaris*), oregano (*Origanum vulgare*)	Xu *et al.* (2006), Vogt-Eisele *et al.* (2007), Choi *et al.* (2016) and Sarkozi *et al.* (2007)
Vanillin	+	NE	NR	–	Vanilla (*Vanilla plantifolia*)	Liibbert *et al.* (2013), Xu *et al.* (2006) and Sarkozi *et al.* (2007)

Table 1. continued

Compound	TRPV1	TRPM8	UCP1	SERCA1	Plants	References
Menthol	–	+	+	NR	Mint (*Mentha longifolia*) and peppermint (*Mentha piperita*)	McKemy, Neuhausser & Julius (2002), Takaishi *et al.* (2016) and Ma *et al.* (2012)
1,8-cineole	NR	+	NR	–	*Eucalyptus* species	Willis *et al.* (2011), Takaishi *et al.* (2012) and Sarkozi *et al.* (2007)
Epigallocatechin-3-gallate (EGCG)	+	NE	+	–	Green tea (*Camellia sinensis*)	Moon *et al.* (2007), Guo *et al.* (2015), Kurogi *et al.* (2012) and Soler, Asensio, & Fernandez-Belda (2012)
Paradol	+	NE	+	NR	Ginger (*Zingiber officinale*)	Sugita *et al.* (2013) and Riera *et al.* (2009)
Zingiberene	+	NR	NR	NR	Ginger (*Zingiber officinale*)	Bandell *et al.* (2004) and Morera *et al.* (2012)
Fucoxanthin	NR	NR	+	NR	*Undaria pinnatifida*	Maeda *et al.* (2007) and Azhar *et al.* (2016)
Aqueous extract	NR	NR	+	NR	*Pinellia ternata*	Kim *et al.* (2006)
Caffeine	+	NE	+	NR	Green tea (*Camellia sinensis*)	Moon *et al.* (2007), Lotteau *et al.* (2013), Daher *et al.* (2009) and Nagatomo & Kubol (2008)

electrolyte loads also require renal excretion mechanisms. Despite fairly widespread existence of halophytic plants, very few herbivore species are specialist consumers. The red vizcacha rat (*Tympanoctomys barreriae*) is only able to consume the leaves of halophytic salt bush (*Atriplex* spp.) due to specialised highly developed renal papillae, allowing production of extremely concentrated urine (Mares *et al.*, 1997). Herbivores without such adaptations (e.g. sheep grazing on salt bush) require higher fresh water intake to allow for urinary excretion of high salt loads (Wilson, 1966). For wild herbivores this excess of fresh water may not be available, leaving a trade-off between food intake and water conservation when food contains PSMs or high electrolyte loads. When body water is being used for evaporative cooling, less still is available for excretion, and hence intake is further constrained at high ambient temperatures.

(4) Plant secondary metabolites that act as mitochondrial uncoupling agents

PSMs that cause up-regulation of UCP1 in BAT, such as some of those listed in Table 1, directly activate thermogenic mechanisms. However, uncoupling can also occur if PSMs act as exogenous mitochondrial uncoupling agents (MUAs) (Fig. 1C). Typically, exogenous MUAs are small, ionisable compounds with moderate to high lipophilicity and extensive charge delocalisation (Wallace & Starkov, 2000; Martineau, 2012). These characteristics result in protonophoric activity by allowing the MUA to pass across the phospholipid bilayer of the inner mitochondrial membrane, picking up hydrogen ions in the inter-membrane space, and releasing them into the mitochondrial matrix (proton shuttling) (Terada, 1990; Wallace & Starkov, 2000; Martineau, 2012). Similar to the action of UCP1, the result is dissipation of the electrochemical gradient generated by the electron transport chain, and uncoupling of cellular respiration from ATP synthesis (Terada, 1990; Wallace & Starkov, 2000; Martineau, 2012). The energy lost in this process is released as heat. Importantly, for both endogenous uncoupling proteins and exogenous uncoupling agents to result in significant thermogenesis, the uncoupled mitochondria need high oxidative capacity. This is commonly observed in the liver, where PSMs are first transported after absorption.

A wide variety of PSMs possess the characteristics necessary to act as exogenous MUAs, including phenols, coumarins, monoterpenes and salicylic acids (Terada, 1990; Martineau, 2012). Very few of these have been tested for uncoupling activity *in vitro* (see Martineau, 2012 for a list of 48 phenolic compounds tested as MUAs), and even fewer *in vivo*, despite their presence in the diets of herbivores. One *in vitro* study found that royleanones from *Salvia officinalis* roots and macrocarpal G from *Eucalyptus viminalis* leaves both caused proton conductivity in artificial bilayer lipid membranes and exerted an uncoupling action on rat liver isolates (Spiridonov *et al.*, 2003). Although the *in vivo* uncoupling effects are unknown, koalas (*Phascolarctos cinereus*) could ingest 2–3 g of macrocarpal G per day when feeding on *E. viminalis* (Moore *et al.*, 2005). Other PSMs that are potent uncouplers include the C-methylated flavonoids myrigalone A, B and G, from the fruits of *Myrica gale* L., which uncoupled oxidative phosphorylation in rat liver mitochondria at double the efficacy of the model proton shuttle uncoupling agent, 2,4-dinitrophenol (DNP) (Mathiesen, Malterud & Sund, 1996).

Few PSMs have been tested for their ability to cause thermogenesis by uncoupling SERCA1, although capsaicin has been shown to uncouple SERCA1 *in vitro* (Mahmmoud, 2008; Table 1). Like MUAs, uncouplers of SERCA1 need to reach a tissue of sufficient oxidative capacity, in an appropriate concentration, to cause significant thermogenesis through uncoupling. For skeletal muscle this means red fibres rather than white fibres, which have higher oxidative capacity and are more numerous in large animals (Emmett & Hochachka, 1981; Hesse, Fischer & Schilling, 2010).

Although the effect of PSMs on thermoregulation and energy balance is rarely considered in nutritional studies, there have been a number of studies that utilise DNP to investigate the impacts of mitochondrial uncoupling on life history. Uncoupling by DNP can reduce exercise capacity (Schlagowski *et al.*, 2014), increase or decrease litter/offspring size (Robert & Bronikowski, 2010; Stier *et al.*, 2014), and alter growth and maturation rates (Ojano-Dirain *et al.*, 2004; Iqbal *et al.*, 2005; Caldeira da Silva *et al.*, 2008; Robert & Bronikowski, 2010; Toyomizu *et al.*, 2011; Salin *et al.*, 2012), and longevity (Barros *et al.*, 2004). In addition, uncoupling by DNP can lead to significant (sometimes fatal) hyperthermia in humans (Grundlingh *et al.*, 2011). Studies are needed that relate feeding or life history to exogenous MUAs found in the natural diet.

In vitro studies alone do not confirm that a PSM will cause *in vivo* mitochondrial uncoupling. For example, the dose of compound needed to induce acute mitochondrial uncoupling may be higher than that ingested naturally. In addition, the absorption, distribution, metabolism and excretion (ADME) of compounds can all play a significant role in the effective dose that a herbivore receives (Forbey *et al.*, 2013). Nevertheless, if MUAs do reach tissues with high oxidative potential, such as the liver, and cause significant thermogenesis through acute uncoupling of oxidative phosphorylation in the mitochondria, the result will be a short-term shift in how much energy is consumed by heat production. Because of reduced mitochondrial efficiency, less of the energy taken in is available for work or storage.

The interactions between diet selection, PSMs, and thermogenesis are clearly complex, and this is likely to be a productive area for future research. The effect of dietary PSMs on heat production *in vivo* could be measured indirectly using respirometry (Thomas, Samson & Bergeron, 1988; Iason & Murray, 1996), whereby the effect would equal the difference between measured metabolic rate after ingestion of a meal containing a PSM minus basal metabolic rate and DIT. An alternative method would be to measure heat production using direct calorimetry. The value of combined respirometry and direct calorimetry is that it

is possible to compare calculated (from O_2 consumption) and measured increases in heat production in response to PSM ingestion. This method could take into account any uncoupling effects of PSMs whereby the increase in heat production exceeds that calculated from the increase in respiration.

VI. PREDICTING RESPONSES TO CLIMATE CHANGE

(1) Strategies to limit hyperthermia associated with feeding

Throughout this review, we have considered evidence that the heat produced by DIT can impede thermoregulation at high ambient temperatures. As hyperthermia has far more immediate detrimental consequences than reduced nutrient intake, it is likely that animals would defend their T_b by altering their feeding ecology at high ambient temperatures. One way in which herbivores could reduce DIT is by down-regulating endogenous uncoupling (i.e. FcT). This would mean that, if the animal was consuming excess overall energy to meet its requirements for protein, the excess might now be stored as fat. The utility of this strategy is limited because selection is unlikely to favour excessive long-term fat storage under most circumstances in the wild, although down-regulation of FcT certainly has the potential to provide a buffer.

Another way to reduce DIT would be to reduce meal size. Since the contribution of ObT to DIT is directly related to the energetic content of the meal, larger meals result in greater DIT, even in an energy-balanced state (i.e. when it cannot be attributed to dietary driven FcT) (Secor, 2009). Reducing meal size would also reduce the amount of thermogenic PSMs ingested. However, should animals choose to decrease meal size, without increasing meal frequency, they may not be able to eat sufficient to meet energy and nutrient requirements for growth and maximal rates of reproduction.

For many herbivores, water is also obtained solely or predominantly from plants, rather than as free water. Therefore, another consequence of decreased food intake is a reduction in water intake. As a case study, consider the koala (*Phascolarctos cinereus*), which obtains both food and water predominantly from *Eucalyptus* leaves. If high ambient temperature drives a reduction in food intake in koalas to minimise DIT, less water will be available for thermoregulation. Koalas use evaporative cooling for heat loss, primarily by panting, but also by licking their forearms, and salivating (Harrop & Degabriele, 1976; Degabriele, Harrop & Dawson, 1978). When ambient temperature approaches body temperature, evaporative cooling is initiated and respiratory rates are increased by up to 20 times (Degabriele & Dawson, 1979). Leaf water, and the physical attributes of trees, can determine tree use by koalas in response to ambient temperature changes (Ellis *et al.*, 2010; Briscoe *et al.*, 2014). Water limitation can also drive the distributions of other arboreal folivore species, as the forest canopy contains few sources of free-standing water, and, in arboreal species, small body sizes preclude significant heat storage (Krockenberger, Edwards & Kanowski, 2012). During heat waves in eastern Australia, koalas descend from trees and search for water along roadsides and in houses, and in several locations, hyperthermia has been blamed for drastic population reductions (Gordon, Brown & Pulsford, 1988*b*; Lunney *et al.*, 2012).

Since some components of the diet make a greater contribution than others to ObT, herbivores could also choose diets that contain different ratios of macronutrients, or lower concentrations of PSMs, in order to reduce DIT. In terms of macronutrients, animals may prefer foods that have a lower protein: carbohydrate ratio, or less fibre. Their ability to do so, of course, depends on the choice and availability of foods with different compositions, as well as their nutritional requirements at the time. For example, if an animal is growing or reproducing (inherently thermogenic processes), it may have higher protein requirements. As a consequence, by reducing intake of thermogenic dietary components such as protein, they may suffer reduced productivity or reproductive success (Atinmo *et al.*, 1976; Sasser *et al.*, 1988; Plavnik & Hurwitz, 1990; DeGabriel *et al.*, 2009). Relatively small changes in the diets of individuals can result in relatively large changes at the population level because of multiplier effects on productivity. The reproductive success and offspring condition of brushtail possums (*Trichosurus vulpecula*) declines dramatically with small changes in leaf digestible nitrogen (DeGabriel *et al.*, 2009). A daily mass gain of 268% was projected in reindeer (*Rangifer tarandus*) following a 14% increase in digestible energy and 27% increase in dry matter intake during selective grazing in summer. A 14% increase in body mass in young female reindeer is sufficient to cause a 35% increase in conception in Autumn (White, 1983). Temperature-driven changes in diet selection by herbivores could equally influence population demographics because "the existence of multiplier effects means that small, difficult-to-detect grazing patterns, nutrient selection processes or even plant avoidance phenomena can strongly influence animal performance" (White, 1983, p. 383).

(2) Shifting plant nutritional quality

The overall nutritional quality of grasses, forbes and trees is predicted to decrease in response to climate change (Robinson, Ryan & Newman, 2012; Rothman *et al.*, 2015; Fig. 3). Protein concentration is likely to decline, but digestible energy may not, and fibre concentration is likely to increase (Bidart-Bouzat & Imeh-Nathaniel, 2008). Several PSMs, including phenolics such as tannins and flavonoids, may increase in concentration, but there are a multitude of interacting factors, including temperature, O_3, soil chemistry, insect herbivory, and precipitation that also influence nutritional quality in glasshouse studies (Bidart-Bouzat & Imeh-Nathaniel, 2008; Craine *et al.*, 2010).

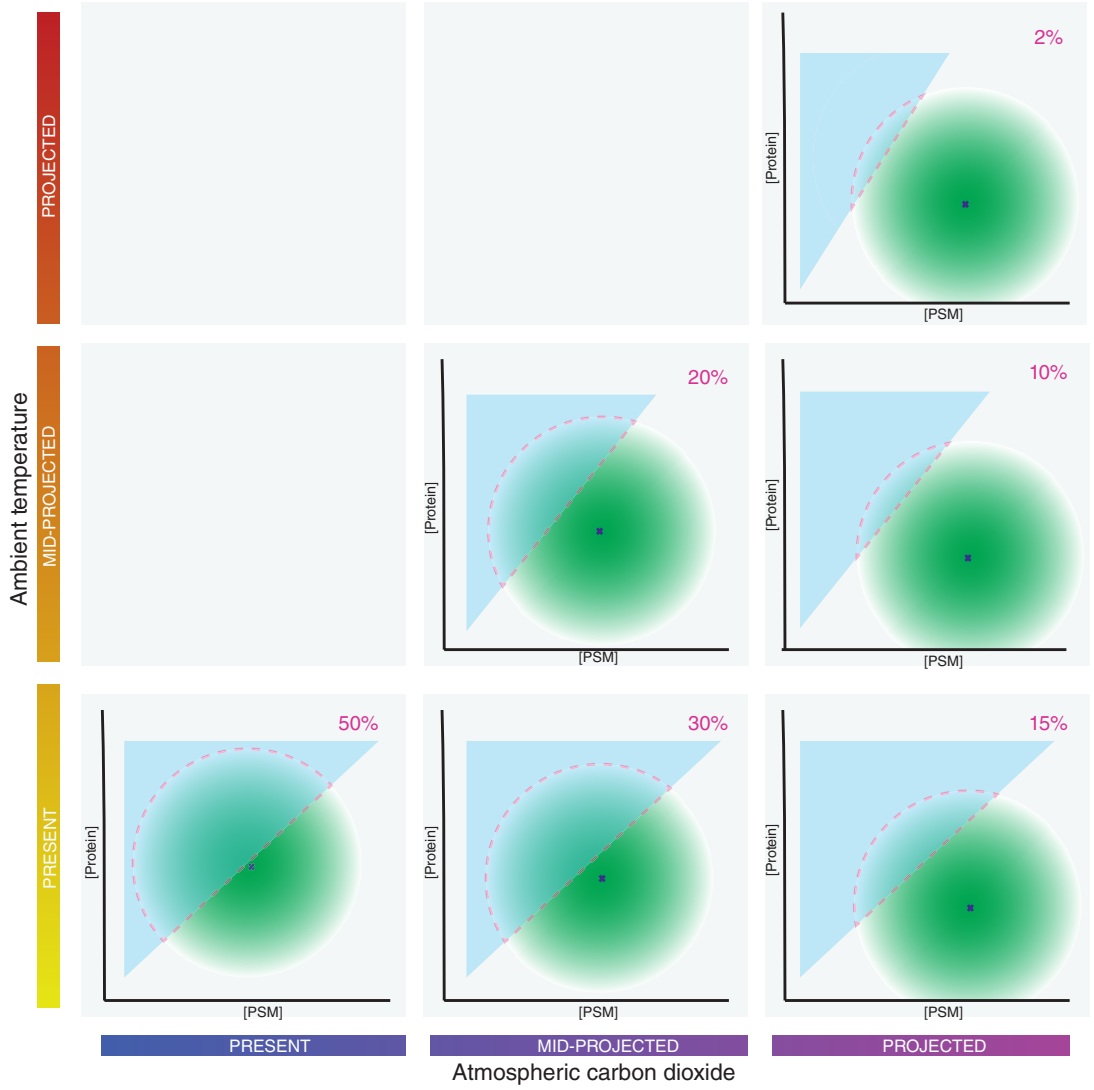

Fig. 3. Conceptual model of the implications of climate change for plant–herbivore interactions. In each panel, the available protein concentration in plants is shown on the *y*-axis, and the plant secondary metabolite (PSM) concentration on the *x*-axis. The green circles represent the range of PSM and protein concentrations available in plants in the environment of the animal. Darker shading indicates more plants. The predicted effect of increasing atmospheric CO_2 on the range of PSM and protein concentrations available in plants is shown from the left to right columns. Blue triangles represent the potential dietary niche of a mammalian herbivore, which could be affected by increasing ambient temperatures (from bottom row to top row). The area outlined by the pink dotted line indicates where herbivory is feasible, because the physiological capacity of the animal overlaps with plant availability. Hypothetical numbers indicate the percentage overlap between the plants available and the dietary niche of the mammalian herbivore. Either an increase in temperature or an increase in atmospheric CO_2 would change the percentage of available plants that are suitable food for herbivores, and together the change is more dramatic.

Translating the effects on plants in small pots to trees in the ground is challenging.

Should PSM concentrations increase, the responses of herbivores to increasing ambient temperatures will be more pronounced, and could dramatically alter the availability of plants that are suitable food for herbivores (Fig. 3). This could be exacerbated by reduced concentrations of protein or available protein, since protein turnover is increased during the metabolism and excretion of many PSMs (Fig. 3). The result of these effects is that herbivore pressure may be skewed towards a smaller number of individual plants (Fig. 3). This, in turn, has the potential to alter a variety of ecosystem processes. Mammalian herbivory has been shown to directly alter plant succession, nutrient flux, primary productivity, insolation, and fire frequency and severity, resulting in flow-on effects to ecosystem dynamics as a whole (Ayers, 1993). Of course, the shift in plant–herbivore dynamics in each system will depend on the species involved, the density of herbivores, the specific requirements of the animal at the time, competition among herbivores, and the distribution of plants with differing chemical and nutrient composition.

The rate of ambient warming, coupled with changes in plant chemistry, will dictate whether changes in feeding behaviour will be sufficient to overcome problems associated with dissipating heat produced by DIT at high ambient temperatures. If animals are unable to find thermal refugia in space or time, or make behavioural or physiological adjustments they will be more susceptible to heat stress or hyperthermia, particularly in heat-wave conditions. If dietary factors threaten thermoregulation, then other activities that generate heat, such as lactation or locomotion may be compromised (Hetem *et al.*, 2016). It is clear that the diet–temperature relationship will be key in predicting ecosystem functioning as a whole, and for understanding physiological challenges faced by mammalian herbivores subject to a changing climate.

VII. CONCLUSIONS

(1) Feeding and thermoregulation in endotherms are linked, but this interaction is often overlooked, particularly in wild herbivores. Increasing ambient temperatures make understanding this relationship more pressing.

(2) The imbalanced nature of herbivore diets and the presence of PSMs which may be thermodisruptive increases the chance of heat stress at high ambient temperatures. Furthermore, detoxification of PSMs can be temperature dependent. Limits to the rate at which heat can be dissipated may require the animal to modulate intake of these compounds to avoid hyperthermia.

(3) Climate change may alter plant–herbivore relationships, as both the physiological tolerance of herbivores to PSMs and the nutritional composition of plants are likely to be affected. Further research is needed to clarify the magnitude and consequences of these complex interactions.

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IX. REFERENCES

Acheson, K. J. (1990). Sympathetic nervous system in the regulation of thermogenesis. In *Hormones and Nutrition in Obesity and Cachexia* (eds M. Müller, E. Danforth, A. Burger and U. Siedentopp), pp. 40–46. Springer, Berlin & Heidelberg.

Ahern, G. P. (2013). Transient receptor potential channels and energy homeostasis. *Trends in Endocrinology and Metabolism* **24**, 554–560.

Aldrich, C. G., Paterson, J. A., Tate, J. L. & Kerley, M. S. (1993). The effects of endophyte-infected tall fescue consumption on diet utilization and thermal regulation in cattle. *Journal of Animal Science* **71**, 164–170.

Anunciado-Koza, R. P., Zhang, J., Ukropec, J., Bajpeyi, S., Koza, R. A., Rogers, R. C., Cefalu, W. T., Mynatt, R. L. & Kozak, L. P. (2011). Inactivation of the mitochondrial carrier SLC25A25 (ATP-Mg^{2+}/Pi transporter) reduces physical endurance and metabolic efficiency in mice. *Journal of Biological Chemistry* **286**, 11659–11671.

Arruda, A. P., Ketzer, L. A., Nigro, M., Galina, A., Carvalho, D. P. & de Meis, L. (2008). Cold tolerance in hypothyroid rabbits: role of skeletal muscle mitochondria and sarcoplasmic reticulum Ca^{2+}-ATPase isoform 1 heat production. *Endocrinology* **149**, 6262–6271.

Asser, A. & Taba, P. (2015). Psychostimulants and movement disorders. *Frontiers in Neurology* **6**, Article 75, 1–13.

Atinmo, T., Baldijao, C., Pond, W. G. & Barnes, R. H. (1976). Prenatal and postnatal protein malnutrition in pigs: effects on growth tate, serum protein and albumin. *Journal of Animal Science* **43**, 606–612.

Au, J., Marsh, K. J., Wallis, I. R. & Foley, W. J. (2013). Whole-body protein turnover reveals the cost of detoxification of secondary metabolites in a vertebrate browser. *Journal of Comparative Physiology B* **183**, 993–1003.

Ayers, M. P. (1993). Plant defense, herbivory and climate change. In *Biotic Interactions and Global Change* (eds P. M. Karieva, J. G. Kingsolver and R. B. Huey), pp. 75–94. Sinauer, Sunderland.

Azhar, Y., Parmar, A., Miller, C. N., Samuels, J. S. & Rayalam, S. (2016). Phytochemicals as novel agents for the induction of browning in white adipose tissue. *Nutrition & Metabolism* **13**, 89.

Babu, G. J., Bhupathy, P., Carnes, C. A., Billman, G. E. & Periasamy, M. (2007). Differential expression of sarcolipin protein during muscle development and cardiac pathophysiology. *Journal of Molecular and Cellular Cardiology* **43**, 215–222.

Bacigalupe, L. D. & Bozinovic, F. (2002). Design, limitations and sustained metabolic rate: lessons from small mammals. *Journal of Experimental Biology* **205**, 2963–2970.

Bal, N. C., Maurya, S. K., Sopariwala, D. H., Sahoo, S. K., Gupta, S. C., Shaikh, S. A., Pant, M., Rowland, L. A., Goonasekera, S. A., Molkentin, J. D. & Periasamy, M. (2012). Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. *Nature Medicine* **18**, 1575–1579.

Bandell, M., Story, G. M., Hwang, S. W., Viswanath, V., Eid, S. R., Petrus, M. J., Earley, T. J. & Patapoutian, A. (2004). Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron* **41**, 849–857.

Barros, M. H., Bandy, B., Tahara, E. B. & Kowaltowski, A. J. (2004). Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **279**, 49883–49888.

Baumgard, L. H. & Rhoads, R. P. (2012). Ruminant nutrition symposium: ruminant production and metabolic responses to heat stress. *Journal of Animal Science* **90**, 1855–1865.

Baziz, H. A., Geraert, P. A., Padilha, J. C. F. & Guillaumin, S. (1996). Chronic heat exposure enhances fat deposition and modifies muscle and fat partition in broiler carcasses. *Poultry Science* **75**, 505–513.

Behrendt, H. J., Germann, T., Gillen, C., Hatt, H. & Jostock, R. (2004). Characterization of the mouse cold-menthol receptor TRPM8 and vanilloid receptor type-1 VR1 using a fluorometric imaging plate reader (FLIPR) assay. *British Journal of Pharmacology* **141**, 737–745.

Belhadj Slimen, I., Najar, T., Ghram, A. & Abdrrabba, M. (2016). Heat stress effects on livestock: molecular, cellular and metabolic aspects, a review. *Journal of Animal Physiology and Animal Nutrition* **100**, 401–412.

Berg, F., Gustafson, U. & Andersson, L. (2006). The uncoupling protein 1 gene (UCP1) is disrupted in the pig lineage: a genetic explanation for poor thermoregulation in piglets. *PLoS Genetics* **2**, 1178–1181.

Bernabucci, U., Bani, P., Ronchi, B., Lacetera, N. & Nardone, A. (1999). Influence of short- and long-term exposure to a hot environment on rumen passage rate and diet digestibility by Friesian heifers. *Journal of Dairy Science* **82**, 967–973.

Berry, M. N., Clark, D. G., Grivell, A. R. & Wallace, P. G. (1985). The contribution of hepatic metabolism to diet-induced thermogenesis. *Metabolism Clinical and Experimental* **34**, 141–147.

Berteaux, D. (2000). Energetic cost of heating ingested food in mammalian herbivores. *Journal of Mammalogy* **81**, 683–690.

Bianca, W. (1965). Reviews of progress of dairy science: section A. Physiology. Cattle in a hot environment. *Journal of Dairy Research* **32**, 291–328.

Bidart-Bouzat, M. G. & Imeh-Nathaniel, A. (2008). Global change effects on plant chemical defenses against insect herbivores. *Journal of Integrative Plant Biology* **50**, 1339–1354.

Bisogno, T., Hanuš, L., De Petrocellis, L., Tchilibon, S., Ponde, D. E., Brandi, I., Moriello, A. S., Davis, J. B., Mechoulam, R. & Di Marzo, V. (2001). Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *British Journal of Pharmacology* **134**, 845–852.

Bligh, J. (1966). Thermosensitivity of the hypothalamus and thermoregulation in mammals. *Biological Reviews of the Cambridge Philosophical Society* **41**, 317–365.

Boutin, S. (1990). Food supplementation experiments with terrestrial vertebrates: patterns, problems, and the future. *Canadian Journal of Zoology* **68**, 203–220.

Briscoe, N. J., Handasyde, K. A., Griffiths, S. R., Porter, W. P., Krockenberger, A. & Kearney, M. R. (2014). Tree-hugging koalas demonstrate a novel thermoregulatory mechanism for arboreal mammals. *Biology Letters* **10**, 20140235.

Brodin, A. (2001). Mass-dependent predation and metabolic expenditure in wintering birds: is there a trade-off between different forms of predation? *Animal Behaviour* **62**, 993–999.

Brown, D. C., Iadarola, M. J., Perkowski, S. Z., Erin, H., Shofer, F., Laszlo, K. J., Olah, Z. & Mannes, A. J. (2005). Physiologic and antinociceptive effects of intrathecal resiniferatoxin in a canine bone cancer model. *Anesthesiology* **103**, 1052–1059.

Caldeira da Silva, C. C., Cerqueira, F. M., Barbosa, L. F., Medeiros, M. H. G. & Kowaltowski, A. J. (2008). Mild mitochondrial uncoupling in mice affects energy metabolism, redox balance and longevity. *Aging Cell* **7**, 552–560.

Campbell, K. L., McIntyre, I. W. & MacArthur, R. A. (2000). Postprandial heat increment does not substitute for active thermogenesis in cold-challenged star-nosed moles (*Condylura cristata*). *Journal of Experimental Biology* **203**, 301–310.

Cannon, B. & Nedergaard, J. (2004). Brown adipose tissue: function and physiological significance. *Physiological Reviews* **84**, 277–359.

Cannon, B. & Nedergaard, J. (2011). Nonshivering thermogenesis and its adequate measurement in metabolic studies. *Journal of Experimental Biology* **214**, 242–253.

Carlsen, M., Lodal, J., Leirs, H. & Jensen, T. S. (1999). The effect of predation risk on body weight in the field vole, *Microtus agrestis*. *Oikos* **87**, 277–285.

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D. & Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**, 816–824.

Chang, S.-S., Wu, M.-L., Deng, J.-F., Lee, C.-C., Chin, T.-F. & Liao, S.-J. (1999). Poisoning by *Datura* leaves used as edible wild vegetables. *Veterinary and Human Toxicology* **41**, 242–245.

Chappell, M. A., Bachman, G. C. & Hammond, K. A. (1997). The heat increment of feeding in house wren chicks: magnitude, duration, and substitution for thermostatic costs. *Journal of Comparative Physiology B* **167**, 313–318.

Chatelain, M., Halpin, C. G. & Rowe, C. (2013). Ambient temperature influences birds' decisions to eat toxic prey. *Animal Behaviour* **86**, 733–740.

Cho, S., Mbiriri, D. T., Shim, K., Lee, A.-L., Oh, S.-J., Yang, J., Ryu, C., Kim, Y.-H., Seo, K.-S., Chae, J.-I., Oh, Y. K. & Choi, N.-J. (2014). The influence of feed energy density and a formulated additive on rumen and rectal temperature in hanwoo steers. *Asian–Australasian Journal of Animal Sciences* **27**, 1652–1662.

Choi, J. H., Kim, S. W., Yu, R. & Yun, J. W. (2016). Monoterpene phenolic compound thymol promotes browning of 3T3-L1 adipocytes. *European Journal of Nutrition*. https://doi.org/10.1007/s00394-016-1273-2.

Clark, W. G. & Lipton, J. M. (1984). Drug-related heatstroke. *Pharmacology & Therapeutics* **26**, 345–388.

Clarke, A. & O'Connor, M. I. (2014). Diet and body temperature in mammals and birds. *Global Ecology and Biogeography* **23**, 1000–1008.

Close, W. H., Mount, L. E. & Start, I. B. (1971). The influence of environmental temperature and plane of nutrition on heat losses from groups of growing pigs. *Animal Production* **13**, 285–294.

Collins, F. G., Mitros, F. A. & Skibba, J. L. (1980). Effect of palmitate on hepatic biosynthetic functions at hyperthermic temperatures. *Metabolism – Clinical and Experimental* **29**, 524–531.

Craine, J. M., Elmore, A. J., Olson, K. C. & Tolleson, D. (2010). Climate change and cattle nutritional stress. *Global Change Biology* **16**, 2901–2911.

Daher, J. P. L., Gover, T. D., Moreira, T. H. V., Lopes, V. G. S. & Weinreich, D. (2009). The identification of a caffeine-induced Ca^{2+} influx pathway in rat primary sensory neurons. *Molecular and Cellular Biochemistry* **327**, 15–19.

Dale, N. M. & Fuller, H. L. (1979). Effect of low temperature, diet density, and pelleting on the preference of broilers for high fat rations. *Poultry Science* **58**, 1337–1339.

Dawson, T. J. & Olson, J. M. (1988). Thermogenic capabilities of the opossum *Monodelphis domestica* when warm and cold acclimated – Similarities between American and Australian marsupials. *Comparative Biochemistry and Physiology A* **89**, 85–91.

Dearing, M. D. (2013). Temperature-dependent toxicity in mammals with implications for herbivores: a review. *Journal of Comparative Physiology B* **183**, 43–50.

Dearing, M. D., Foley, W. J. & McLean, S. (2005). The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. *Annual Review of Ecology Evolution and Systematics* **36**, 169–189.

Dearing, M. D., Mangione, A. M. & Karasov, W. H. (2001). Plant secondary compounds as diuretics: an overlooked consequence. *American Zoologist* **41**, 890–901.

DeGabriel, J. L., Moore, B. D., Foley, W. J. & Johnson, C. N. (2009). The effects of plant defensive chemistry on nutrient availability predict reproductive success in a mammal. *Ecology* **90**, 711–719.

Degabriele, R. & Dawson, T. J. (1979). Metabolism and heat balance in an arboreal marsupial, the koala (*Phascolarctos cinereus*). *Journal of Comparative Physiology* **134**, 293–301.

Degabriele, R., Harrop, C. J. F. & Dawson, T. J. (1978). Water metabolism of the koala (*Phascolarctos cinereus*). In *The Ecology of Arboreal Folivores* (ed. G. G. Montgomery), pp. 163–172. Smithsonian Institution, Washington.

Delorme, M. & Thomas, D. W. (1996). Nitrogen and energy requirements of the short-tailed fruit bat (*Carollia perspicillata*): fruit bats are not nitrogen constrained. *Journal of Comparative Physiology B* **166**, 427–434.

De Petrocellis, L., Vellani, V., Schiano-Moriello, A., Marini, P., Marini, P., Magherini, P. C., Orlando, P. & Di Marzo, V. (2008). Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. *Journal of Pharmacology and Experimental Therapeutics* **325**, 1007–1015.

Devendra, C. & Leng, R. A. (2011). Feed resources for animals in Asia: issues, strategies for use, intensification and integration for increased productivity. *Asian–Australasian Journal of Animal Sciences* **24**, 303–321.

Egan, A. R. (1989). Living with, and overcoming limits to, feeding value of high-fibre roughages. In *Draught Animals in Rural Development* (eds D. Hoffman, J. Nari and R. J. Petheram), pp. 176–180. Australian Centre for International Agricultural Research (ACIAR), Canberra.

Ellis, W., Melzer, A., Clifton, I. & Carrick, F. (2010). Climate change and the koala *Phascolarctos cinereus*: water and energy. *Australian Zoologist* **35**, 369–377.

Else, P. L., Turner, N. & Hulbert, A. J. (2004). The evolution of endothermy: role for membranes and molecular activity. *Physiological and Biochemical Zoology* **77**, 950–958.

Emmett, B. & Hochachka, P. W. (1981). Scaling of oxidative and glycolytic-enzymes in mammals. *Respiration Physiology* **45**, 261–272.

Espinoza, R. E., Wiens, J. J. & Tracy, C. R. (2004). Recurrent evolution of herbivory in small, cold-climate lizards: breaking the ecophysiological rules of reptilian herbivory. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 16819–16824.

Faulconnier, Y., Bonnet, M., Bocquier, F., Leroux, C. & Chilliard, Y. (2001). Effects of photoperiod and feeding level on adipose tissue and muscle lipoprotein lipase activity and mRNA level in dry non-pregnant sheep. *British Journal of Nutrition* **85**, 299–306.

Feldmann, H. M., Golozoubova, V., Cannon, B. & Nedergaard, J. (2009). UCP1 ablation induces obesity and abolishes diet-induced thermogenesis in mice exempt from thermal stress by living at thermoneutrality. *Cell Metabolism* **9**, 203–209.

Felton, A. M., Felton, A., Raubenheimer, D., Simpson, S. J., Foley, W. J., Wood, J. T., Wallis, I. R. & Lindenmayer, D. B. (2009). Protein content of diets dictates the daily energy intake of a free-ranging primate. *Behavioral Ecology* **20**, 685–690.

Foley, W. J., McLean, S. & Cork, S. J. (1995). Consequences of biotransformation of plant secondary metabolites on acid-base metabolism in mammals – a final common pathway? *Journal of Chemical Ecology* **21**, 721–743.

Forbey, J. S., Dearing, M. D., Gross, E. M., Orians, C. M., Sotka, E. E. & Foley, W. J. (2013). A pharm-ecological perspective of terrestrial and aquatic plant-herbivore interactions. *Journal of Chemical Ecology* **39**, 465–480.

Forbey, J. S. & Foley, W. J. (2009). PharmEcology: a pharmacological approach to understanding plant–herbivore interactions. *Integrative and Comparative Biology* **49**, 267–273.

Fromme, T. & Klingenspor, M. (2011). Uncoupling protein 1 expression and high-fat diets. *American Journal of Physiology* **300**, R1–R8.
Fu, S. J., Xie, X. J. & Cao, Z. D. (2005). Effect of meal size on postprandial metabolic response in southern catfish (*Silurus meridionalis*). *Comparative Biochemistry and Physiology A* **140**, 445–451.
Gaughan, J. B., Mader, T. L., Holt, S. M. & Lisle, A. (2008). A new heat load index for feedlot cattle. *Journal of Animal Science* **86**, 226–234.
Gavva, N. R. (2008). Body temperature maintenance as the predominant function of the vanilloid receptor TRPV1. *Trends in Pharmacological Sciences* **29**, 550–557.
Gordon, C. J., Johnstone, A. F. M. & Aydin, C. (2014). Thermal stress and toxicity. *Comprehensive Physiology* **4**, 995–1016.
Gordon, C. J., Mohler, F. S., Watkinson, W. P. & Rezvani, A. H. (1988*a*). Temperature regulation in laboratory mammals following acute toxic insult. *Toxicology* **53**, 161–178.
Gordon, G., Brown, A. S. & Pulsford, T. (1988*b*). A koala (*Phascolarctos cinereus* Goldfuss) population crash during drought and heatwave conditions in south-western Queensland. *Australian Journal of Ecology* **13**, 451–461.
Grundlingh, J., Dargan, P. I., El-Zanfaly, M. & Wood, D. M. (2011). 2,4-dinitrophenol (DNP): a weight loss agent with significant acute toxicity and risk of death. *Journal of Medical Toxicology* **7**, 205–212.
Grynkiewicz, G. & Gadzikowska, M. (2008). Tropane alkaloids as medicinally useful natural products and their synthetic derivatives as new drugs. *Pharmacological Reports* **60**, 439–463.
Guo, B. C., Wei, J., Su, K. H., Chiang, A. N., Zhao, J. F., Chen, H. Y., Shyue, S. K. & Lee, T. S. (2015). Transient receptor potential vanilloid type 1 is vital for (-)-epigallocatechin-3-gallate mediated activation of endothelial nitric oxide synthase. *Molecular Nutrition & Food Research* **59**, 646–657.
Hall, G. M., Lucke, J. N., Lovell, R. & Lister, D. (1980). Porcine malignant hyperthermia. VII: Hepatic metabolism. *British Journal of Anaesthesia* **52**, 11–17.
Hammond, K. & Diamond, J. (1994). Limits to dietary nutrient intake and intestinal nutrient uptake in lactating mice. *Physiological Zoology* **67**, 282–303.
Hammond, K. A. & Diamond, J. (1992). An experimental test for a ceiling on sustained metabolic rate in lactating mice. *Physiological Zoology* **65**, 952–977.
Harms, M. & Seale, P. (2013). Brown and beige fat: development, function and therapeutic potential. *Nature Medicine* **19**, 1252–1263.
Harrop, C. J. F. & Degabriele, R. (1976). Digestion and nitrogen metabolism in the koala, *Phascolarctos cinereus*. *Australian Journal of Zoology* **24**, 201–215.
Herd, R. M., Oddy, V. H. & Richardson, E. C. (2004). Biological basis for variation in residual feed intake in beef cattle. 1. Review of potential mechanisms. *Australian Journal of Experimental Agriculture* **44**, 423–430.
Herrera, G. L., Gutierrez, E., Hobson, K. A., Altube, B., Díaz, W. G. & Sánchez-Cordero, V. (2002). Sources of assimilated protein in five species of New World frugivorous bats. *Oecologia* **133**, 280–287.
Hesse, B., Fischer, M. S. & Schilling, N. (2010). Distribution pattern of muscle fiber types in the perivertebral musculature of two different sized species of mice. *Anatomical Record-Advances in Integrative Anatomy and Evolutionary Biology* **293**, 446–463.
Hetem, R. S., Maloney, S. K., Fuller, A. & Mitchell, D. (2016). Heterothermy in large mammals: inevitable or implemented? *Biological Reviews* **91**, 187–205.
Higginson, A. D., McNamara, J. M. & Houston, A. I. (2012). The starvation-predation trade-off predicts trends in body size, muscularity and adiposity between and within taxa. *American Naturalist* **179**, 338–350.
Huang, X., Hancock, D. P., Gosby, A. K., McMahon, A. C., Solon, S. M. C., Le Couteur, D. G., Conigrave, A. D., Raubenheimer, D. & Simpson, S. J. (2013). Effects of dietary protein to carbohydrate balance on energy intake, fat storage, and heat production in mice. *Obesity* **21**, 85–92.
Hulbert, A. J. & Else, P. L. (1990). The cellular basis of endothermic metabolism – a role for leaky membranes. *News in Physiological Sciences* **5**, 25–28.
Iadarola, M. J. & Mannes, A. (2011). The vanilloid agonist resiniferatoxin for interventional-based pain control. *Current Topics in Medicinal Chemistry* **11**, 2171–2179.
Iason, G. R. & Murray, A. H. (1996). The energy costs of ingestion of naturally occurring nontannin plant phenolics by sheep. *Physiological Zoology* **69**, 532–546.
Ibragic, S. & Sofic, E. (2015). Chemical composition of various *Ephedra* species. *Bosnian Journal of Basic Medical Sciences* **15**, 21–27.
Iqbal, M., Pumford, N. R., Tang, Z. X., Lassiter, K., Ojano-Dirain, C., Wing, T., Cooper, M. & Bottje, W. (2005). Compromised liver mitochondrial function and complex activity in low feed efficient broilers are associated with higher oxidative stress and differential protein expression. *Poultry Science* **84**, 933–941.
Jastroch, M., Withers, K. W., Taudien, S., Frappell, P. B., Helwig, M., Fromme, T., Hirschberg, V., Heldmaier, G., McAllan, B. M., Firth, B. T., Burmester, T., Platzer, M. & Klingenspor, M. (2008). Marsupial uncoupling protein 1 sheds light on the evolution of mammalian nonshivering thermogenesis. *Physiological Genomics* **32**, 161–169.
Jeffry, J. A., Yu, S.-Q., Sikand, P., Parihar, A., Evans, M. S. & Premkumar, L. S. (2009). Selective targeting of TRPV1 expressing sensory nerve terminals in the spinal cord for long lasting analgesia. *PLoS ONE* **4**, e7021.
Jobling, M. & Davies, P. S. (1980). Effects of feeding on metabolic rate, and the specific dynamic action in plaice, *Pleuronectes platessa* L. *Journal of Fish Biology* **16**, 629–638.
Johnson, M. S. & Thomson, S. C. (2001). Limits to sustained energy intake III. Effects of concurrent pregnancy and lactation in *Mus musculus*. *Journal of Experimental Biology* **30**, 267–271.
Johnson, M. S., Thomson, S. C. & Speakman, J. R. (2001). Limits to sustained energy intake. I. Lactation in the laboratory mouse *Mus musculus*. *Journal of Experimental Biology* **204**, 1925–1935.
de Jonge, H. W., van der Wiel, C. W., Eizema, K., Weijs, W. A. & Everts, M. E. (2006). Presence of SERCA and calcineurin during fetal development of porcine skeletal muscle. *Journal of Histochemistry and Cytochemistry* **54**, 641–648.
Kaneko, Y. & Szallasi, A. (2014). Transient receptor potential (TRP) channels: a clinical perspective. *British Journal of Pharmacology* **171**, 2474–2507.
Kaplanski, J. & Ben-Zvi, Z. (1980). Effect of chronic heat exposure on in-vitro drug metabolism in the rat. *Life Sciences* **26**, 639–642.
Keplinger, M. L., Lanier, G. E. & Deichmann, W. B. (1959). Effects of environmental temperature on the acute toxicity of a number of compounds in rats. *Toxicology and Applied Pharmacology* **1**, 156–161.
Kida, R., Yoshida, H., Murakami, M., Shirai, M., Hashimoto, O., Kawada, T., Matsui, T. & Funaba, M. (2016). Direct action of capsaicin in brown adipogenesis and activation of brown adipocytes. *Cell Biochemistry and Function* **34**, 34–41.
Kim, N., Nam, M., Kang, M. S., Lee, J. O., Lee, Y. W., Hwang, G. S. & Kim, H. S. (2017). Piperine regulates UCP1 through the AMPK pathway by generating intracellular lactate production in muscle cells. *Scientific Reports* **7**, 41066.
Kim, Y.-J., Shin, Y.-O., Ha, Y.-W., Lee, S., Oh, J.-K. & Kim, Y. S. (2006). Anti-obesity effect of *Pinellia ternata* extract in Zucker rats. *Biological and Pharmaceutical Bulletin* **29**, 1278–1281.
Kjelstrup, S., de Meis, L., Bedeaux, D. & Simon, J. M. (2008). Is the Ca^{2+}-ATPase from sarcoplasmic reticulum also a heat pump? *European Biophysics Journal with Biophysics Letters* **38**, 59–67.
Kobayashi, A., Osaka, T., Namba, Y., Inoue, S., Lee, T. H. & Kimura, S. (1998). Capsaicin activates heat loss and heat production simultaneously and independently in rats. *American Journal of Physiology* **275**, R92–R98.
Kobayashi, M., Shoji, N. & Ohizumi, Y. (1987). Gingerol, a novel cardiotonic agent, activates the Ca^{2+}-pumping ATPase in skeletal and cardiac sarcoplasmic reticulum. *Biochimica et Biophysica Acta* **903**, 96–102.
Koh, W. U., Choi, S. S., Kim, J. H., Yoon, H. J., Ahn, H. S., Lee, S. K., Leem, J. G., Song, J. G. & Shin, J. W. (2016). The preventive effect of resiniferatoxin on the development of cold hypersensitivity induced by spinal nerve ligation: involvement of TRPM8. *BMC Neuroscience* **17**, 38.
Komissarov, I. V. (1964). Mechanism of action of ephedrine. *Bulletin of Experimental Biology and Medicine* **57**, 442–445.
Konarzewski, M. & Diamond, J. (1994). Peak sustained metabolic rate and its individual variation in cold-stressed mice. *Physiological Zoology* **67**, 1186–1212.
Kozak, L. P. (2010). Brown fat and the myth of diet-induced thermogenesis. *Cell Metabolism* **11**, 263–267.
Krauss, S., Zhang, C.-Y. & Lowell, B. B. (2005). The mitochondrial uncoupling-protein homologues. *Nature Reviews Molecular Cellular Biology* **6**, 248–261.
Krockenberger, A. K., Edwards, W. & Kanowski, J. (2012). The limit to the distribution of a rainforest marsupial folivore is consistent with the thermal intolerance hypothesis. *Oecologia* **168**, 889–899.
Król, E., Murphy, M. & Speakman, J. R. (2007). Limits to sustained energy intake. X. Effects of fur removal on reproductive performance in laboratory mice. *Journal of Experimental Biology* **210**, 4233–4243.
Kurnath, P. & Dearing, M. D. (2013). Warmer ambient temperatures depress liver function in a mammalian herbivore. *Biology Letters* **9**, 20130562.
Kurnath, P., Merz, N. D. & Dearing, M. D. (2016). Ambient temperature influences tolerance to plant secondary compounds in a mammalian herbivore. *Proceedings of the Royal Society of London Series B* **283**, 20152387.
Kurogi, M., Miyashita, M., Emoto, Y., Kubo, Y. & Saitoh, O. (2012). Green tea polyphenol epigallocatechin gallate activates TRPA1 in an intestinal enteroendocrine cell line, STC-1. *Chemical Senses* **37**, 167–177.
Laurien-Kehnen, C. & Trillmich, F. (2003). Lactation performance of guinea pigs (*Cavia porcellus*) does not respond to experimental manipulation of pup demands. *Behavioral Ecology and Sociobiology* **53**, 145–152.
Lima, S. L. (1986). Predation risk and unpredictable feeding conditions: determinants of body mass in birds. *Ecology* **67**, 377–385.
Lincoln, G. A., Rhind, S. M., Pompolo, S. & Clarke, I. J. (2001). Hypothalamic control of photoperiod-induced cycles in food intake, body weight, and metabolic hormones in rams. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **281**, R76–R90.
Little, A. G. & Seebacher, F. (2014). The evolution of endothermy is explained by thyroid hormone-mediated responses to cold in early vertebrates. *Journal of Experimental Biology* **217**, 1642–1648.
Lotteau, S., Ducreux, S., Romestaing, C., Legrand, C. & Van Coppenolle, F. (2013). Characterization of functional TRPV1 channels in the sarcoplasmic reticulum of mouse skeletal muscle. *PLoS ONE* **8**, e58673.
Lowell, B. B. & Spiegelman, B. M. (2000). Towards a molecular understanding of adaptive thermogenesis. *Nature* **404**, 652–660.

Lübbert, M., Kyereme, J., Schöbel, N., Beltrán, L., Wetzel, C. H. & Hatt, H. (2013). Transient receptor potential channels encode volatile chemicals sensed by rat trigeminal ganglion neurons. *PLoS ONE* **8**, e77998.

Lunney, D., Crowther, M. S., Wallis, I. R., Foley, W. J., Lemon, J., Wheeler, R., Madani, G., Orscheg, C., Griffith, J. E., Krockenberger, M. & Retamales, M. (2012). Koalas and climate change: a case study on the Liverpool Plains, north-west New South Wales. In *Wildlife and Climate Change: Towards Robust Conservation Strategies for Australian Fauna* (eds D. Lunney and P. Hutchings), pp. 150–168. Royal Zoological Society of NSW, Mosman.

Ma, S., Yu, H., Zhao, Z., Luo, Z., Chen, J., Ni, Y., Jin, R., Ma, L., Wang, P., Zhu, Z., Li, L., Zhong, J., Liu, D., Nilius, B. & Zhu, Z. (2012). Activation of the cold-sensing TRPM8 channel triggers UCP1-dependent thermogenesis and prevents obesity. *Journal of Molecular Cell Biology* **4**, 88–96.

Ma, S. W. Y., Foster, D. O., Nadeau, B. E. & Triandafillou, J. (1988). Absence of increased oxygen consumption in brown adipose tissue of rats exhibiting "cafeteria" diet-induced thermogenesis. *Canadian Journal of Physiology and Pharmacology* **66**, 1347–1354.

MacLeod, R., MacLeod, C. D., Learmonth, J. A., Jepson, P. D., Reid, R. J., Deaville, R. & Pierce, G. J. (2007). Mass-dependent predation risk and lethal dolphin–porpoise interactions. *Proceedings of the Royal Society of London Series B* **274**, 2587–2593.

Macpherson, L. J., Hwang, S. W., Miyamoto, T., Dubin, A. E., Patapoutian, A. & Story, G. M. (2006). More than cool: promiscuous relationships of menthol and other sensory compounds. *Molecular and Cellular Neuroscience* **32**, 335–343.

Maeda, H., Hosokawa, M., Sashima, T., Funayama, K. & Miyashita, K. (2007). Effect of medium-chain triacylglycerols on anti-obesity effect of fucoxanthin. *Journal of Oleo Science* **56**, 615–621.

Mahmmoud, Y. A. (2008). Capsaicin stimulates uncoupled ATP hydrolysis by the sarcoplasmic reticulum calcium pump. *Journal of Biological Chemistry* **283**, 21418–21426.

Mahmmoud, Y. A. & Gaster, M. (2012). Uncoupling of sarcoplasmic reticulum Ca^{2+}-ATPase by N-arachidonoyl dopamine. Members of the endocannabinoid family as thermogenic drugs. *British Journal of Pharmacology* **166**, 2060–2069.

Mall, S., Broadbridge, R., Harrison, S. L., Gore, M. G., Lee, A. G. & East, J. M. (2006). The presence of sarcolipin results in increased heat production by Ca^{2+}-ATPase. *Journal of Biological Chemistry* **281**, 36597–36602.

Marder, J., Eylath, U., Moskovitz, E. & Sharir, R. (1990). The effect of heat exposure on blood chemistry of the hyperthermic rabbit. *Comparative Biochemistry and Physiology A* **97**, 245–247.

Mares, M. A., Ojeda, R. A., Borghi, C. E., Giannoni, S. M., Diaz, G. B. & Braun, J. K. (1997). How desert rodents overcome halophytic plant defenses. *Bioscience* **47**, 699–704.

Martineau, L. C. (2012). Simple thermodynamic model of unassisted proton shuttle uncoupling and prediction of activity from calculated speciation, lipophilicity, and molecular geometry. *Journal of Theoretical Biology* **303**, 33–61.

Mathiesen, L., Malterud, K. E. & Sund, R. B. (1996). Uncoupling of respiration and inhibition of ATP synthesis in mitochondria by C-methylated flavonoids from *Myrica gale* L. *European Journal of Pharmaceutical Sciences* **4**, 373–379.

Maurya, S. K., Bal, N. C., Sopariwala, D. H., Pant, M., Rowland, L. A., Shaikh, S. A. & Periasamy, M. (2015). Sarcolipin is a key determinant of the basal metabolic rate, and its overexpression enhances energy expenditure and resistance against diet-induced obesity. *Journal of Biological Chemistry* **290**, 10840–10849.

McKemy, D. D., Neuhausser, W. M. & Julius, D. (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **416**, 52–58.

McNamara, F. N., Randall, A. & Gunthorpe, M. J. (2005). Effects of piperine, the pungent component of black pepper, at the human vanilloid receptor (TRPV1). *British Journal of Pharmacology* **144**, 781–790.

de Meis, L. (2002). Ca^{2+}-ATPases (SERCA): energy transduction and heat production in transport ATPases. *Journal of Membrane Biology* **188**, 1–9.

de Meis, L., Arruda, A. P. & Carvalho, D. P. (2005). Role of sarco/endoplasmic reticulum Ca^{2+}-ATPase in thermogenesis. *Bioscience Reports* **25**, 181–190.

Miller, D. S. & Payne, P. R. (1962). Weight maintenance and food intake. *Journal of Nutrition* **78**, 255–262.

Molyneux, R. J. & Panter, K. E. (2009). Alkaloids toxic to livestock. In *The Alkaloids: Chemistry and Biology* (ed. G. A. Cordell), pp. 143–216. Academic Press, San Diego.

Moon, H.-S., Lee, H.-G., Choi, Y.-J., Kim, T.-G. & Cho, C.-S. (2007). Proposed mechanisms of (−)-epigallocatechin-3-gallate for anti-obesity. *Chemico-Biological Interactions* **167**, 85–98.

Moore, B. D., Foley, W. J., Wallis, I. R., Cowling, A. & Handasyde, K. A. (2005). *Eucalyptus* foliar chemistry explains selective feeding by koalas. *Biology Letters* **1**, 64–67.

Morera, E., De Petrocellis, L., Morera, L., Schiano-Moriello, A., Nalli, M., Di Marzo, V. & Ortar, G. (2012). Synthesis and biological evaluation of [6]-gingerol analogues as transient receptor potential channel TRPV1 and TRPA1 modulators. *Bioorganic and Medicinal Chemistry Letters* **22**, 1674–1677.

Müller, S., Balaz, M., Stefanicka, P., Varga, L., Amri, E.-Z., Ukropec, J., Wollscheid, B. & Wolfrum, C. (2016). Proteomic analysis of human brown adipose tissue reveals utilization of coupled and uncoupled energy expenditure pathways. *Scientific Reports* **6**, 30030.

Nagatomo, K. & Kubol, Y. (2008). Caffeine activates mouse TRPA1 channels but suppresses human TRPA1 channels. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 17373–17378.

Nagy, T. R., Gower, B. A. & Stetson, M. H. (1995). Endocrine correlates of seasonal body mass dynamics in the collared lemming (*Dicrostonyx groenlandicus*). *American Zoologist* **35**, 246–258.

Ojano-Dirain, C. P., Iqbal, M., Cawthon, D., Swonger, S., Wing, T., Cooper, M. & Bottje, W. (2004). Determination of mitochondrial function and site-specific defects in electron transport in duodenal mitochondria in broilers with low and high feed efficiency. *Poultry Science* **83**, 1394–1403.

Okon, E. E. (1980). Histological changes of the interscapular brown adipose tissue in *Eidolon helvum* in relation to diurnal activities of the bats. In *Fifth International Bat Research Conference, 1980 New Mexico* (eds D. E. Wilson and A. L. Gardner). Texas Tech Press, Lubbock.

Oliver, A. J. & King, D. R. (1983). The influence of ambient temperatures on the susceptibility of mice, guinea pigs and possums to compound 1080. *Australian Wildlife Research* **10**, 297–301.

Ominski, K. H., Kennedy, A. D., Wittenberg, K. M. & Nia, S. A. M. (2002). Physiological and production responses to feeding schedule in lactating dairy cows exposed to short-term, moderate heat stress. *Journal of Dairy Science* **85**, 730–737.

Ono, K., Tsukamoto-Yasui, M., Hara-Kimura, Y., Inoue, N., Nogusa, Y., Okabe, Y., Nagashima, K. & Kato, F. (2011). Intragastric administration of capsiate, a transient receptor potential channel agonist, triggers thermogenic sympathetic responses. *Journal of Applied Physiology* **110**, 789–798.

Park, C. K., Kim, K., Jung, S. J., Kim, M. J., Ahn, D. K., Hong, S. D., Kim, J. S. & Oh, S. B. (2009). Molecular mechanism for local anesthetic action of eugenol in the rat trigeminal system. *Pain* **144**, 84–94.

Parray, H. A. & Yun, J. W. (2016). Cannabidiol promotes browning in 3T3-L1 adipocytes. *Molecular and Cellular Biochemistry* **416**, 131–139.

Parry, M. L., Canziani, O. F., Palutikof, J. P., Van der Linden, P. J. & Hanson, C. E. (2007). *Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge.

Pearce, L. V., Petukhov, P. A., Szabo, T., Kedei, N., Bizik, F., Kozikowski, A. P. & Blumberg, P. M. (2004). Evodiamine functions as an agonist for the vanilloid receptor TRPV1. *Organic and Biomolecular Chemistry* **2**, 2281–2286.

Perrigo, G. (1987). Breeding and feeding strategies in deer mice and house mice when females are challenged to work for their food. *Animal Behaviour* **35**, 1298–1316.

Plavnik, I. & Hurwitz, S. (1990). Performance of broiler chickens and turkey poults subjected to feed restriction or to feeding of low-protein or low-sodium diets at an early age. *Poultry Science* **69**, 945–952.

Pough, F. H. (1973). Lizard energetics and diet. *Ecology* **54**, 837–844.

Premkumar, L. S. (2014). Transient receptor potential channels as targets for phytochemicals. *ACS Chemical Neuroscience* **5**, 1117–1130.

Prunier, A., de Bragança, M. M. & Le Dividich, J. (1997). Influence of high ambient temperature on performance of reproductive sows. *Livestock Production Science* **52**, 123–133.

Raisinghani, M., Pabbidi, R. M. & Premkumar, L. S. (2005). Activation of transient receptor potential vanilloid 1 (TRPV1) by resiniferatoxin. *Journal of Physiology* **567**, 771–786.

Raubenheimer, D. & Simpson, S. J. (1997). Integrative models of nutrient balancing: application to insects and vertebrates. *Nutrition Research Reviews* **10**, 151–179.

Renaudeau, D., Collin, A., Yahav, S., de Basilio, V., Gourdine, J. L. & Collier, R. J. (2012). Adaptation to hot climate and strategies to alleviate heat stress in livestock production. *Animal* **6**, 707–728.

Renaudeau, D., Quiniou, N. & Dubois, S. (2002). Effects of high ambient temperature and dietary protein level on feeding behavior of multiparous lactating sows. *Animal Research* **51**, 227–243.

Rezende, E. L. & Bacigalupe, L. D. (2015). Thermoregulation in endotherms: physiological principles and ecological consequences. *Journal of Comparative Physiology B* **185**, 709–727.

Rhoads, R. P., Baumgard, L. H., Suagee, J. K. & Sanders, S. R. (2013). Nutritional interventions to alleviate the negative consequences of heat stress. *Advances in Nutrition* **4**, 267–276.

Riera, C. E., Menozzi Smarrito, C., Affolter, M., Michlig, S., Munari, C., Robert, F., Vogel, H., Simon, S. A. & Le Coutre, J. (2009). Compounds from Sichuan and Melegueta peppers activate, covalently and non-covalently, TRPA1 and TRPV1 channels. *British Journal of Pharmacology* **157**, 1398–1409.

Robert, K. A. & Bronikowski, A. M. (2010). Evolution of senescence in nature: physiological evolution in populations of garter snake with divergent life histories. *American Naturalist* **175**, 147–159.

Robinson, E. A., Ryan, G. D. & Newman, J. A. (2012). A meta-analytical review of the effects of elevated CO_2 on plant-arthropod interactions highlights the importance of interacting environmental and biological variables. *New Phytologist* **194**, 321–336.

Rosen, D. A. S. & Trites, A. W. (2003). No evidence for bioenergetic interaction between digestion and thermoregulation in Steller Sea Lions *Eumetopias jubatus*. *Physiological and Biochemical Zoology* **76**, 899–906.

Ross, L. G., Mckinney, R. W., Cardwell, S. K., Fullarton, J. G., Roberts, S. E. J. & Ross, B. (1992). The effects of dietary protein content, lipid content and ration level on oxygen consumption and specific dynamic action in *Oreochromis niloticus* L. *Comparative Biochemistry and Physiology A* **103**, 573–578.

Rothman, J. M., Chapman, C. A., Struhsaker, T. T., Raubenheimer, D., Twinomugisha, D. & Waterman, P. G. (2015). Long-term declines in nutritional quality of tropical leaves. *Ecology* **96**, 873–878.

Rothwell, N. J. & Stock, M. J. (1979). Effects of continuous and discontinuous periods of cafeteria feeding on body weight, resting oxygen consumption and noradrenaline sensitivity in the rat. *Journal of Physiology* **291**, 59.

Rowland, L. A., Bal, N. C. & Periasamy, M. (2015). The role of skeletal-muscle-based thermogenic mechanisms in vertebrate endothermy. *Biological Reviews of the Cambridge Philosophical Society* **90**, 1279–1297.

Saito, M. (2015). Capsaicin and related food ingredients reducing body fat through the activation of TRP and brown fat thermogenesis. In *Advances in Food and Nutrition Research* (ed. J. Henry), pp. 1–28. Academic Press, Waltham.

Salin, K., Luquet, E., Rey, B., Roussel, D. & Voituron, Y. (2012). Alteration of mitochondrial efficiency affects oxidative balance, development and growth in frog (*Rana temporaria*) tadpoles. *Journal of Experimental Biology* **215**, 863–869.

Sarkozi, S., Almassy, J., Lukacs, B., Dobrosi, N., Nagy, G. & Jona, I. (2007). Effect of natural phenol derivatives on skeletal type sarcoplasmic reticulum Ca^{2+}-ATPase and ryanodine receptor. *Journal of Muscle Research and Cell Motility* **28**, 167–174.

Sasser, R. G., Williams, R. J., Bull, R. C., Ruder, C. A. & Falk, D. G. (1988). Postpartum reproductive performance in crude protein-restricted beef cows: return to estrus and conception. *Journal of Animal Science* **66**, 3033–3039.

Schlagowski, A. I., Singh, F., Charles, A. L., Ramamoorthy, T. G., Favret, F., Piquard, F., Geny, B. & Zoll, J. (2014). Mitochondrial uncoupling reduces exercise capacity despite several skeletal muscle metabolic adaptations. *Journal of Applied Physiology* **116**, 364–375.

Scholander, P. F. (1955). Evolution of climatic adaptation in homeotherms. *Evolution* **9**, 15–26.

Schwartz, M. W., Figlewicz, D. P., Baskin, D. G., Woods, S. C. & Porte, D. Jr. (2008). Insulin in the brain: a hormonal regulator of energy balance. *Endocrine Reviews* **13**, 387–414.

Secor, S. M. (2009). Specific dynamic action: a review of the postprandial metabolic response. *Journal of Comparative Physiology B* **179**, 1–56.

Secor, S. M. & Boehm, M. (2006). Specific dynamic action of ambystomatid salamanders and the effects of meal size, meal type, and body temperature. *Physiological and Biochemical Zoology* **79**, 720–735.

Secor, S. M. & Diamond, J. (1997). Determinants of the postfeeding metabolic response of Burmese pythons, *Python molurus*. *Physiological Zoology* **70**, 202–212.

Selescu, T., Ciobanu, A. C., Dobre, C., Reid, G. & Babes, A. (2013). Camphor activates and sensitizes transient receptor potential melastatin 8 (TRPM8) to cooling and icilin. *Chemical Senses* **38**, 563–575.

Settivari, R. S., Evans, T. J., Eichen, P. A., Rottinghaus, G. E. & Spiers, D. E. (2008). Short- and long-term responses to fescue toxicosis at different ambient temperatures. *Journal of Thermal Biology* **33**, 213–222.

Settivari, R. S., Evans, T. J., Yarru, L. P., Eichen, P. A., Sutovsky, P., Rottinghaus, G. E., Antoniou, E. & Spiers, D. E. (2009). Effects of short-term heat stress on endophytic ergot alkaloid-induced alterations in rat hepatic gene expression. *Journal of Animal Science* **87**, 3142–3155.

Silva, J. E. (2006). Thermogenic mechanisms and their hormonal regulation. *Physiological Reviews* **86**, 435–464.

Silva, J. E. & Rabelo, R. (1997). Regulation of the uncoupling protein gene expression. *European Journal of Endocrinology* **136**, 251–264.

Simpson, S. J. & Raubenheimer, D. (2001). The geometric analysis of nutrient-allelochemical interactions: a case study using locusts. *Ecology* **82**, 422–439.

Smith, W. S., Broadbridge, R., East, J. M. & Lee, A. G. (2002). Sarcolipin uncouples hydrolysis of ATP from accumulation of Ca^{2+} by the Ca^{2+}-ATPase of skeletal-muscle sarcoplasmic reticulum. *Biochemical Journal* **361**, 277–286.

Soler, F., Asensio, M. C. & Fernandez-Belda, F. (2012). Inhibition of the intracellular Ca^{2+} transporter SERCA (Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase) by the natural polyphenol epigallocatechin-3-gallate. *Journal of Bioenergetics and Biomembranes* **44**, 597–605.

Sørensen, A., Mayntz, D., Raubenheimer, D. & Simpson, S. J. (2008). Protein-leverage in mice: the geometry of macronutrient balancing and consequences for fat deposition. *Obesity* **16**, 566–571.

Speakman, J. R. (2000). The cost of living: field metabolic rates of small mammals. *Advances in Ecological Research* **30**, 176–297.

Speakman, J. R. (2014). If body fatness is under physiological regulation, then how come we have an obesity epidemic? *Physiology* **29**, 88–98.

Speakman, J. R. & Król, E. (2010). Maximal heat dissipation capacity and hyperthermia risk: neglected key factors in the ecology of endotherms. *Journal of Animal Ecology* **79**, 726–746.

Speakman, J. R. & Westerterp, K. R. (2013). A mathematical model of weight loss under total starvation: evidence against the thrifty-gene hypothesis. *Disease Models and Mechanisms* **6**, 236–251.

Spiers, D. E., Evans, T. J. & Rottinghaus, G. E. (2005). Interaction between thermal stress and Fescue toxicosis: animal models and new perspectives. In *Neotyphodium in Cool-Season Grasses* (eds C. A. Roberts, C. P. West and D. E. Spiers), pp. 243–270. Blackwell Publishing Ltd, Oxford.

Spiridonov, N. A., Arkhipov, V. V., Foigel, A. G., Shipulina, L. D. & Fomkina, M. G. (2003). Protonophoric and uncoupling activity of royleanones from *Salvia officinalis* and euvimals from *Eucalyptus viminalis*. *Phytotherapy Research* **17**, 1228–1230.

Stadnyk, A. N. & Glezos, J. D. (1983). Drug-induced heat stroke. *Canadian Medical Association Journal* **128**, 957–959.

Stevens, C. E. & Hume, I. D. (1998). Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiological Reviews* **78**, 393–427.

Stier, A., Bize, P., Roussel, D., Schull, Q., Massemin, S. & Criscuolo, F. (2014). Mitochondrial uncoupling as a regulator of life-history trajectories in birds: an experimental study in the zebra finch. *Journal of Experimental Biology* **217**, 3579–3589.

Stock, M. J. (1999). Gluttony and thermogenesis revisited. *International Journal of Obesity* **23**, 1105–1117.

Sugita, J., Yoneshiro, T., Hatano, T., Aita, S., Ikemoto, T., Uchiwa, H., Iwanaga, T., Kameya, T., Kawai, Y. & Saito, M. (2013). Grains of paradise (*Aframomum melegueta*) extract activates brown adipose tissue and increases whole-body energy expenditure in men. *British Journal of Nutrition* **110**, 733–738.

Takaishi, M., Fujita, F., Uchida, K., Yamamoto, S., Sawada, M., Hatai, C., Shimizu, M. & Tominaga, M. (2012). 1,8-cineole, a TRPM8 agonist, is a novel natural antagonist of human TRPA1. *Molecular Pain* **8**, 86.

Takaishi, M., Uchida, K., Suzuki, Y., Matsui, H., Shimada, T., Fujita, F. & Tominaga, M. (2016). Reciprocal effects of capsaicin and menthol on thermosensation through regulated activities of TRPV1 and TRPM8. *Journal of Physiological Sciences* **66**, 143–155.

Terada, H. (1990). Uncouplers of oxidative phosphorylation. *Environmental Health Perspectives* **87**, 213–218.

Teulier, L., Rouanet, J.-L., Letexier, D., Romestaing, C., Belouze, M., Rey, B., Duchamp, C. & Roussel, D. (2010). Cold-acclimation-induced non-shivering thermogenesis in birds is associated with upregulation of avian UCP but not with innate uncoupling or altered ATP efficiency. *Journal of Experimental Biology* **213**, 2476–2482.

Thomas, D. W., Samson, C. & Bergeron, J. M. (1988). Metabolic costs associated with the ingestion of plant phenolics by *Microtus pennsylvanicus*. *Journal of Mammalogy* **69**, 512–515.

Toyomizu, M., Kikusato, M., Kawabata, Y., Azad, M. A. K., Inui, E. & Amo, T. (2011). Meat-type chickens have a higher efficiency of mitochondrial oxidative phosphorylation than laying-type chickens. *Comparative Biochemistry and Physiology A* **159**, 75–81.

Turbill, C., Ruf, T., Mang, T. & Arnold, W. (2011). Regulation of heart rate and rumen temperature in red deer: effects of season and food intake. *Journal of Experimental Biology* **214**, 963–970.

Vangheluwe, P., Schuermans, M., Zador, E., Waelkens, E., Raeymaekers, L. & Wuytack, F. (2005). Sarcolipin and phospholamban mRNA and protein expression in cardiac and skeletal muscle of different species. *Biochemical Journal* **389**, 151–159.

Varga, A., Németh, J., Szabó, Á., McDougall, J. J., Zhang, C., Elekes, K., Pintér, E., Szolcsányi, J. & Helyes, Z. (2005). Effects of the novel TRPV1 receptor antagonist SB366791 in vitro and in vivo in the rat. *Neuroscience Letters* **385**, 137–142.

Vassallo, S. U. & Delaney, K. A. (1989). Pharmacologic effects on thermoregulation – Mechanisms of drug-related heatstroke. *Journal of Toxicology-Clinical Toxicology* **27**, 199–224.

Vogt-Eisele, A. K., Weber, K., Sherkheli, M. A., Vielhaber, G., Panten, J., Gisselmann, G. & Hatt, H. (2007). Monoterpenoid agonists of TRPV3. *British Journal of Pharmacology* **151**, 530–540.

Wallace, K. B. & Starkov, A. A. (2000). Mitochondrial targets of drug toxicity. *Annual Review of Pharmacology and Toxicology* **40**, 353–388.

Wang, T., Wang, Y., Kontani, Y., Kobayashi, Y., Sato, Y., Mori, N. & Yamashita, H. (2008). Evodiamine improves diet-induced obesity in a uncoupling protein-1-independent manner: involvement of antiadipogenic mechanism and extracellularly regulated kinase/mitogen-activated protein kinase signaling. *Endocrinology* **149**, 358–366.

Wang, X., Miyares, R. L. & Ahern, G. P. (2005). Oleoylethanolamide excites vagal sensory neurones, induces visceral pain and reduces short-term food intake in mice via capsaicin receptor TRPV1. *Journal of Physiology* **564**, 541–547.

Wang, Z., Ying, Z., Bosy-Westphal, A., Zhang, J., Schautz, B., Later, W., Heymsfield, S. B. & Müller, M. J. (2010). Specific metabolic rates of major organs and tissues across adulthood: evaluation by mechanistic model of resting energy expenditure. *American Journal of Clinical Nutrition* **92**, 1369–1377.

Weihe, W. H. (1973). Effect of temperature on action of drugs. *Annual Review of Pharmacology and Toxicology* **13**, 409–425.

Welbergen, J. A., Klose, S. M., Markus, N. & Eby, P. (2008). Climate change and the effects of temperature extremes on Australian flying-foxes. *Proceedings of the Royal Society of London Series B* **275**, 419–425.
West, J. W. (1999). Nutritional strategies for managing the heat-stressed dairy cow. *Journal of Animal Science* **77**, 21–35.
Westerterp, K. R. (2004). Diet-induced thermogenesis. *Nutrition and Metabolism* **1**, 5.
Wheelock, J. B., Rhoads, R. P., VanBaale, M. J., Sanders, S. R. & Baumgard, L. H. (2010). Effects of heat stress on energetic metabolism in lactating Holstein cows. *Journal of Dairy Science* **93**, 644–655.
White, R. G. (1983). Foraging patterns and their multiplier effects on productivity of northern ungulates. *Oikos* **40**, 377–384.
Willis, D. N., Liu, B., Ha, M. A., Jordt, S.-E. & Morris, J. B. (2011). Menthol attenuates respiratory irritation responses to multiple cigarette smoke irritants. *FASEB Journal* **25**, 4434–4444.
Wilson, A. D. (1966). Intake and excretion of sodium by sheep fed on species of *Atriplex* (saltbush) and *Kochia* (bluebush). *Australian Journal of Agricultural Research* **17**, 155–163.
Xu, H., Blair, N. T. & Clapham, D. E. (2005). Camphor activates and strongly desensitizes the transient receptor potential vanilloid subtype 1 channel in a vanilloid-independent mechanism. *Journal of Neuroscience* **25**, 8924–8937.
Xu, H., Delling, M., Jun, J. C. & Clapham, D. E. (2006). Oregano, thyme and clove-derived flavors and skin sensitizers activate specific TRP channels. *Nature Neuroscience* **9**, 628–635.
Yang, B. H., Piao, Z. G., Kim, Y. B., Lee, C. H., Lee, J. K., Park, K., Kim, J. S. & Oh, S. B. (2003). Activation of vanilloid receptor 1 (VR1) by eugenol. *Journal of Dental Research* **82**, 781–785.
Zurbenko, I. & Luo, M. (2015). Surface humidity changes in different temporal scales. *American Journal of Climate Change* **4**, 226–238.

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Chapter 2

Reduced hepatic detoxification in marsupial herbivores following moderate heat exposure

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Abstract

Ambient temperature influences the foraging behaviour of herbivores, partly due to changes in energy requirements and the heat produced during digestion. Plant secondary metabolites (PSMs) can also have temperature-dependent effects on the feeding behaviour of herbivores. Research in pharmacology and ecological physiology suggests that ambient temperature affects the rate at which PSMs and other xenobiotics are metabolised by herbivores. This has crucial implications for wild herbivores that regularly ingest xenobiotics. Here, we measured the functional clearance time of an anaesthetic agent in two species of marsupial folivores, the common brushtail possum (*Trichosurus vulpecula*) and the common ringtail possum (*Pseudocheirus peregrinus*) at three different temperatures. We found a positive correlation between functional clearance times and ambient temperature in both species after the possums were exposed to temperatures of 10°C, 18°C, and 26°C for seven days. There was no effect of temperature on functional clearance times in *T. vulpecula* after an overnight exposure to temperatures of 10°C and 26°C. These data provide physiological evidence for temperature-dependent toxicity (TDT) in marsupials, reflected in reduced liver function at warmer ambient temperatures. However, it is important to consider exposure time in understanding the effects of ambient temperature. As global temperatures continue to rise, TDT is likely to become increasingly important in many species of herbivores, both wild and domesticated, that ingest PSMs.

Keywords: temperature-dependent toxicity, Alfaxalone, thermoregulation, herbivory, functional clearance time, plant secondary metabolites

Introduction

Herbivory is the most common feeding strategy found across mammalian species, and is an important determinant of ecosystem function (Foley and Moore 2005). Feeding decisions by herbivores influence plant chemistry and species diversity within ecosystems (Provenza et al. 2003; Twigg et al. 2003). Most browsers ingest significant amounts of plant secondary metabolites (PSMs) in every meal (Dearing et al. 2005; Freeland and Janzen 1974; Li et al. 2006; Marsh et al. 2014). Many of these PSMs are absorbed and subsequently modified for excretion, primarily through hepatic biotransformation, but in some cases also via microbial metabolism (Dearing et al. 2005; Kohl et al. 2014; Krieger et al. 1971). The rate at which an herbivore metabolizes PSMs can determine the rate at which it consumes food, and therefore obtains critical nutrients (Marsh et al. 2006a; Torregrossa and Dearing 2009). Detoxification rates can be influenced by a number of factors, including the nutritional state of the animal (Marsh et al. 2006a), the induction state of detoxification enzymes (e.g. through previous exposure to PSMs; Boyle and McLean 2004; Pass et al. 1999) and the availability of co-factors for PSM conjugation (Marsh et al. 2005). Recently, there has been interest in how an extrinsic factor, ambient temperature, could influence hepatic detoxification, and hence the dietary strategies of mammalian herbivores (Dearing 2013; Kurnath and Dearing 2013; Kurnath et al. 2016).

Ambient temperature has long been known to influence hepatic detoxification rates in laboratory rodents (Keplinger et al. 1959), with warmer temperatures increasing the toxicity of many xenobiotics (Gordon et al. 2014; Kaplanski and Ben-Zvi 1980; Keplinger et al. 1959). This phenomenon is known as temperature-dependent toxicity (TDT; Dearing 2013). Mechanistically, TDT results from the need to balance thermal homeostasis with detoxification of PSMs or other xenobiotics, because the liver is both the main site for biotransformation and the largest heat-producing organ (Berry et al. 1985; Wang et al. 2010). Thus, mammals are presented with a physiological trade-off between these two processes at higher ambient temperatures (Beale et al. 2018). Importantly, TDT has been proposed to explain some of the variation in food intake by herbivores eating diets containing PSMs at different ambient temperatures. For example, herbivorous rodents (genus *Neotoma*) had greater tolerance for ecologically

relevant PSMs at cooler temperatures than at warmer temperatures (Kurnath et al. 2016). Such an interaction between ambient temperature and toxicity will likely also influence the diet selection of other herbivore species.

Measuring rates of detoxification of PSMs in wild herbivores is challenging for a number of reasons. For example, the detoxification pathways of many PSMs are unknown, and can be complex (Marsh et al. 2005). Even when pathways have been characterised, it is often difficult to collect regular blood or urine samples from wild animals. Furthermore, traditional measures of toxicity, such as LD_{50}, are ethically and ecologically questionable when studying wildlife species. Previous studies have used anaesthetic agents, such as hexobarbital, as proxy compounds to determine the functional clearance time of hepatically metabolised xenobiotics, since their effects are easily observed in hypnotic state assays (Dearing et al. 2006; Kurnath and Dearing 2013; Sasaki 1994). During such assays, the total time for which animals sleep indicates the functional clearance time of the drug. Hypnotic state assays have the benefit of being non-lethal, and allowing the comparison of multiple treatments within one individual. Hypnotic state assays may provide a practical alternative to investigate the influence of factors like ambient temperature on rates of hepatic metabolism.

In the present study, we aimed to test whether ambient temperature influences hepatic detoxification rates in two folivorous marsupial species, the common brushtail possum (*Trichosurus vulpecula*) and common ringtail possum (*Pseudocheirus peregrinus*). Common ringtail possums are small (700-900g) folivores that predominantly eat tree leaves, including significant amounts of *Eucalyptus* (Hermsen et al. 2016; Pahl 1987a). Common brushtail possums are larger (2-3 kg) generalist herbivores that consume *Eucalyptus* leaves and a range of leaves, flowers and fruits from other plant species (McDowell and McLeod 2007). These possum species are good candidates to investigate temperature-dependent rates of hepatic metabolism because the feeding behaviour of both is influenced by specific PSMs (e.g. Lawler et al. 1998; Marsh et al. 2003a; Marsh et al. 2003b).

We measured the functional clearance time of a modern anaesthetic agent, Alfaxalone, using a hypnotic state assay. Hexobarbital (the drug that is most commonly used in hypnotic state assays e.g. Dearing et al. 2006; Kurnath and Dearing 2013) is a highly

alkaline barbiturate drug (pH 11.5), and is thus an irritant when injected intraperitoneally, intramuscularly, or subcutaneously (Barron and Dundee 1961). In contrast, Alfaxalone is a neuroactive steroid used routinely in veterinary medicine as a safe and effective anaesthetic (Warne et al. 2015). Alfaxalone undergoes hepatic phase 1 (P450 dependent) and phase 2 (glucuronide and sulphate conjugation dependent) metabolism (Warne et al. 2015), making it an ideal proxy compound for PSMs. Thus, to determine the impact of ambient temperature on hepatic detoxification, we tested the rate at which possums metabolised Alfaxalone after either one week or < 24 h exposure to various ambient temperatures.

Different exposure times to ambient temperatures may influence the liver in different ways. For instance, short-term exposure to a particular temperature, such as might be experienced during a heat wave, can induce fast acting and often temporary changes at the cellular level (Horowitz 1998). Conversely, longer exposure periods can cause structural modifications at the organ level that are slower to change, including alterations to vasculature and overall liver size (Nespolo et al. 2002). Such structural changes might reflect, for example, seasonal differences in temperature acclimation. Understanding whether animals respond differently to various exposure times will help to elucidate whether ecologically relevant temperature fluctuations are likely to affect hepatic metabolism.

Methods

Animal capture and housing

Twelve adult male common brushtail possums (*Trichosurus vulpecula;* mean body mass = 3.04 kg, sd = 0.38 kg) were caught on the Australian National University (ANU) campus, Canberra, Australia, in March of 2015, using cage traps baited with apple and peanut butter. These possums were used for the week-long exposure experiment. Another 12 adult male brushtail possums were caught on the ANU campus in January 2016 for the short exposure experiment (mean body mass = 3.03 kg, sd = 0.25 kg). The health and age (by tooth wear; Pahl 1987b; Winter 1980) of all possums was checked following capture.

Following capture, brushtail possums were housed individually in aviaries (90 x 70 x 152 cm) inside constant temperature rooms (initially set to 18 °C) on a 12 h:12 h light:dark cycle with a 30 min change in the intensity of light to simulate dusk and dawn. All possums were provided with wooden platforms with two sides (18 x 18 x 34 cm) to provide some shelter, but to prevent any temperature buffering. Following capture, brushtail possums were provided with fresh fruit and mixed leaves daily. Prior to the experiment, diets were gradually changed to a prepared diet (42.5% chopped apple, 28% chopped banana, 10% pureed carrot, 6% ground rice hulls, 6% rolled oats, 4% ground lucerne chaff, 1.67% acid casein, 1.25% vegetable oil, 0.3% NaCl, 0.25% dicalcium phosphate, and 0.03% vitamin and mineral mix (Nutrimol, Vitagran), all on a wet matter basis) that was prepared fresh daily. Fresh water was always available.

Ten adult male and one non-lactating adult female common ringtail possums (*Pseudocheirus peregrinus*) were caught by hand in the Black Mountain Nature Reserve, Canberra, Australia, in March 2015 (mean body mass = 749 g, sd = 90 g). They were housed inside constant temperature rooms (18 °C, 12 h:12 h light:dark cycle) individually in wire cages (66 x 65 x 92 cm). Ringtail possums were provided with wooden nest boxes (15 x 15 x 20 cm) with one side formed from mesh to minimise temperature buffering while permitting the animals to shelter. Ringtail possums were fed daily with *Eucalyptus rossii* foliage. Water was provided *ad libitum*.

Week-long exposure experiment

Four possums of each species were randomly allocated to each of three ambient temperature treatments (10 °C, 18 °C and 26 °C). These temperatures were chosen based on respirometry data indicating that 10 °C is below the lower critical temperature (LCT), 18 °C is around the LCT and 26 °C is just below the upper critical temperature (UCT) of the thermoneutral zone (TNZ) of ringtail possums (Beale et al. unpublished data), and predictions for the TNZ of brushtail possums (Porter and Kearney 2009; Riek and Geiser 2013).

Possums were maintained at the experimental temperatures for one week. After this, the clearance time assay was conducted over two days, between 0900 h and 1200 h, to ensure that all individuals were tested during a similar period of the circadian cycle. Thus, on the seventh day, two possums of each species from each temperature were

administered 5 $mg.kg^{-1}$ of Alfaxalone (Jurox, Rutherford NSW, Australia) intramuscularly. The chosen dose was at the lower end of the range recommended for fast acting and short-term veterinary anaesthesia (Miller and Folwer 2015). Animals were held in a hessian (brushtail possums) or calico (ringtail possums) bag under observation until drowsy, and then removed and placed on their backs in an observation cage. The length of time that each possum stayed on its back until righting itself was taken as the "sleep time", or the functional drug clearance time. On the eighth day, the procedure was repeated for the remaining possums from each temperature. Two experimenters were always involved, with one as primary handler and timer, and one as primary injector and observer. The clearance time assays were repeated over three rounds in a randomised crossover design such that each possum was tested at all three experimental temperatures. The order of administration was randomised by temperature between rounds to account for any effects of the time of administration. There was always a wash-out period of seven days between rounds to allow hepatic enzymes to return to baseline levels (Pass et al. 2001).

Short exposure experiment

All 12 brushtail possums were held at 18 °C (the midway point between the two experimental temperatures) for at least one week prior to the start of the experiment. Then, at 1600 h, the ambient temperature was reset to either 10 °C (six possums) or 26 °C (six possums). The temperature of both rooms stabilised within 1 h. The clearance time assay was conducted between 0800 h and 1200 h the following morning, when possums had been exposed to the experimental temperatures for between 17-20 h. The procedure for the assay was otherwise identical to the week-long exposure assay. Possums were returned to 18 °C for another week, after which the clearance time assay was repeated at the other temperature.

Statistical analyses

Six possums were either mis-injected or were too aroused from handling prior to injection, and did not fall asleep. These data points (one ringtail possum, four brushtail possums from the week-long exposure experiment, and one from the short exposure experiment) were excluded from statistical analysis.

Data from the week-long temperature exposure experiments were analyzed in R studio using a generalized linear mixed model (Packages lme4, lmeTest,) to test the effect of temperature on functional clearance times. We made an initial model with temperature and species and an interaction as the fixed effects. The model also included three random effects; individual possum identity, day on which the anaesthetic was administered, and the order in which each possum was subjected to the temperature treatments. Using an ANOVA (type III) we determined that the interaction was not significant, so the same model without the interaction term was compared using an ANOVA with a null model containing only random effects.

Data from the short-term exposure experiment were also analysed using a generalized linear mixed model with the temperature as the fixed effect, and possum identity, and the day of anaesthetic administration as random effects. The model with temperature as the fixed effect was compared to a null model with random variables using an ANOVA.

Results

Week-long exposure experiment

Both temperature ($F(2) = 23.954$, $P < 0.001$) and possum species ($F(1) = 57.457$, $P < 0.001$) significantly affected functional clearance times, and our model with temperature and possum species as fixed effects was significantly better than the null model ($\chi2(3) = 61.25$, $P <0.001$). Functional clearance times were 43% longer at 26 °C compared to 10 °C after possums were exposed for 7-8 days (Figure 1). Ringtail possums always had a shorter functional clearance time than did brushtail possums (Figure 1).

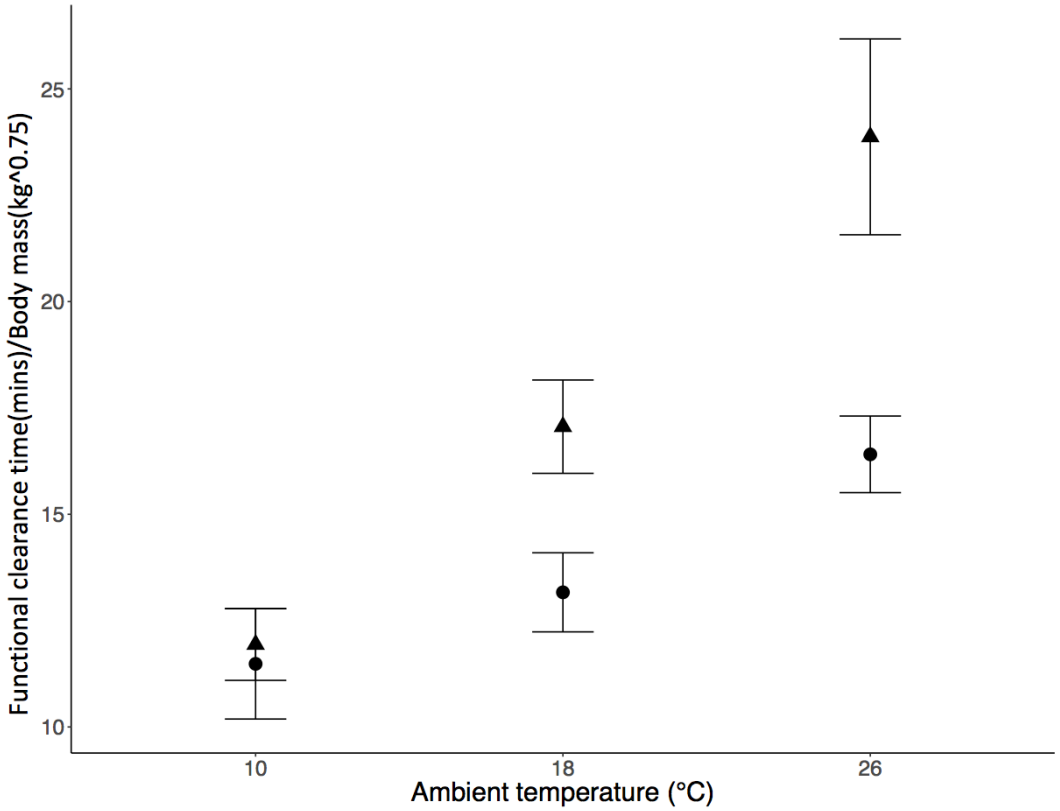

Fig. 1 Functional clearance times (mean ± SE) for ringtail possums (triangles, n=11) and brushtail possums (circles, n=9) injected with 5 mg.kg^{-1} Alfaxalone, expressed relative to body mass to the power of 0.75, following seven to eight days of exposure to different ambient temperatures

Short exposure experiment

There was no significant effect of ambient temperature on functional clearance times in common brushtail possums after a short temperature exposure period ($\chi 2(1) = 1.26$, $p = 0.26$; Figure 2). Mean functional clearance times between 10 °C and 26 °C differed by only 1.6 minutes (Figure 2), compared to the 10-minute average difference after a week-long exposure period (Figure 1).

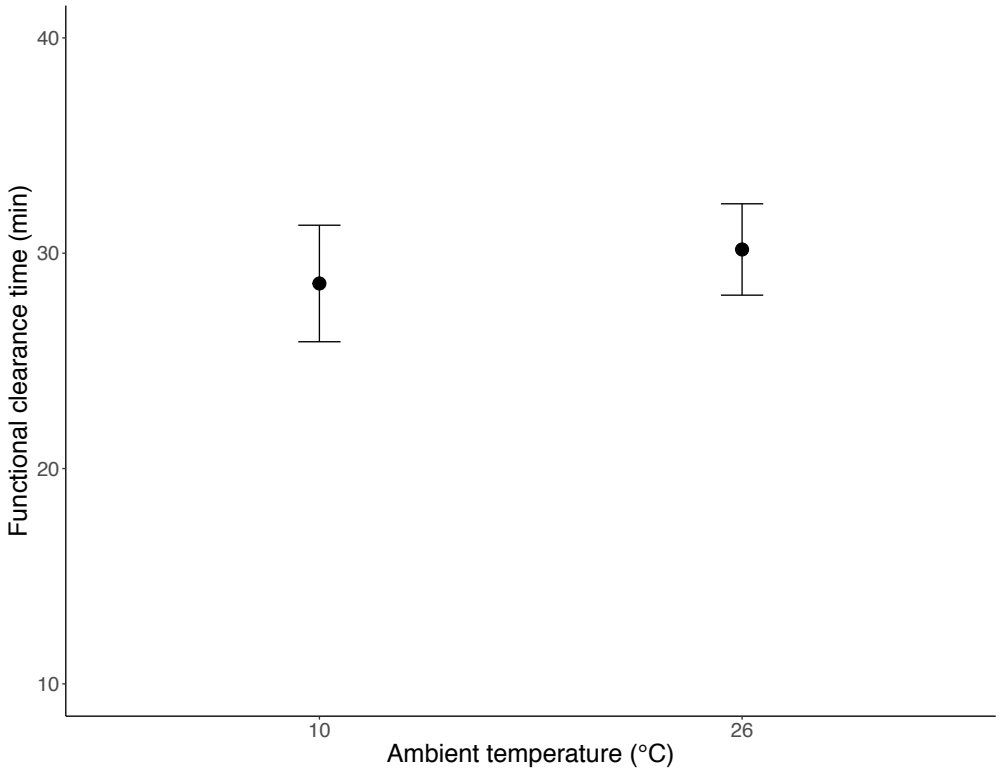

Fig. 2 Mean ± SE functional clearance times for *T. vulpecula* (N=10) treated with Alfaxalone following short-term (<24h) exposure to different ambient temperatures

Discussion

Detoxification of plant secondary metabolites presents a physiological challenge for mammalian herbivores, and our results demonstrate that this challenge is exacerbated by high ambient temperatures. We found a positive relationship between the functional clearance time of the anaesthetic agent, Alfaxalone, and ambient temperature when possums were exposed to temperatures for one week. This indicates that liver function in possums is reduced after one week of exposure to warmer ambient temperatures compared to cooler ambient temperatures, providing evidence for temperature-dependent toxicity (TDT). However, there was no difference in the functional clearance time of Alfaxalone after a short exposure period to two different ambient temperatures in the common brushtail possum, suggesting that TDT in possums depends on the length of exposure to ambient temperatures. TDT is a phenomenon that has been well documented in pharmacological studies with laboratory rodents. However, this study

reiterates the ecological and evolutionary importance of TDT for wild herbivores eating diets rich in plant secondary metabolites (PSMs).

Our results have important implications for the feeding decisions of the common brushtail and the common ringtail possum. The difference in functional clearance times between the ambient temperature treatments suggests that possums experiencing warmer ambient temperatures will, over time, likely also have prolonged PSM clearance rates. Alfaxalone, and some of the PSMs found in *Eucalyptus,* are metabolized by similar hepatic pathways, namely oxidation by Cytochrome p450 enzymes and conjugation to glucuronic acid (McLean and Duncan 2006; Warne et al. 2015). Possums are able to effectively regulate their intake of PSMs to remain below the limits of their detoxification capacity and avoid toxicity (Marsh et al. 2006a; Marsh et al. 2005; Marsh et al. 2006b). Our results demonstrate that the rate of hepatic detoxification is influenced by ambient temperature, such that the upper limit of detoxification capacity seems to shift down as ambient temperature increases, and thus, plants containing PSMs essentially become more toxic. Therefore, to avoid toxicity at warmer temperatures, possums may need to regulate their PSM intake around a lower set point by selectively consuming plants with lower concentrations of PSMs, or by reducing total food intake. These sorts of temperature-mediated shifts in PSM tolerance and intake have been documented in herbivorous rodents (Dearing et al. 2008; Kurnath et al. 2016) and in omnivorous birds (Chatelain et al. 2013).

Three complementary mechanisms likely underpin the longer functional clearance times documented at warmer ambient temperatures. Firstly, hepatic metabolism, including detoxification, leads to heat production within the body core (Ohnhaus and Tilvis 1976). Excess heat must be dissipated if mammals are to avoid hyperthermia. However, dissipation of excess heat becomes more costly for mammals, in terms of energy and water loss, when ambient temperatures are high. The rate at which excess heat can be dissipated therefore limits physiological activities that generate heat, a phenomenon referred to as heat dissipation limitation (Beale et al. 2018; Speakman and Król 2010). In the current study, longer functional clearance times may have been the result of down-regulation of hepatic activity to reduce the production of excess heat, and potential hyperthermia.

Secondly, a change in the total number of hepatic enzymes may explain temperature-mediated shifts in liver metabolism. Previous work investigating the effect of ambient temperature on hepatic gene expression found that genes associated with xenobiotic metabolism, antioxidative mechanisms, and immune function were down-regulated after short-term heat-shock in laboratory rodents (Settivari et al. 2009). In addition, woodrats that were housed at an ambient temperature only 5 °C warmer than conspecifics, had reduced liver expression of genes associated with metabolic function (e.g. P450 (cytochrome) oxidoreductase and glutathione S-transferase M2; Connors et al. 2017).

The third element that may affect functional clearance times is the redistribution of blood. Blood is shunted away from the periphery in cold conditions to conserve heat, and is directed towards the periphery in warm conditions to increase heat loss (Hales et al. 1979). Changes in blood distribution could alter the rate at which xenobiotics are exposed to liver enzymes. Modification of blood flow patterns, such as vasodilation at the periphery, can occur rapidly as ambient temperature changes (Charkoudian 2010). However, there was no difference in functional clearance time between 10 °C and 26 °C after short-term exposure to these temperatures. This suggests that short-term changes in blood flow (if they occurred) were not sufficient to alter rates of Alfaxalone metabolism in possums. There may also, however, be longer-term effects of changes in blood flow. Reduced hepatic blood flow over time results in hepatocyte apoptosis until liver mass and blood flow are matched, resulting in a decrease in liver size (Lautt 2007; Nespolo et al. 2002). Liver size in rodents is known to decrease after only 4-5 days at temperatures that are 10-16°C warmer than standard laboratory conditions (Settivari et al. 2008; Toloza et al. 1991). It is therefore possible that possums exposed to warmer temperatures for one week experienced a reduction in liver size, resulting in longer functional clearance times. Contrary to this, expansion of plasma volume to compensate for peripheral vasodilation has also been observed following long-term heat exposure, and this improvement in organ temperature regulation is a feature of heat adaptation (Périard et al. 2016). Therefore, while we have proposed potential mechanisms for reduced functional clearance times following long exposure to warm temperatures, we recognise a need for future research to clarify these mechanisms.

It is interesting to note that rates of hepatic metabolism can be reduced even at seemingly mild ambient temperatures, within the TNZ, and within natural environmental temperature ranges. A previous study with herbivorous woodrats (genus *Neotoma*) reported similar findings (Kurnath and Dearing 2013). A crucial difference between the woodrat study and our results is the effect of exposure time to temperature treatments on hepatic metabolism. While the woodrat study found a significant reduction in functional clearance time after a three-hour exposure period, the present study found no difference in functional clearance time after a 17-20 hour exposure period to experimental temperatures. One reason for this could be the large difference in body mass between the species. Common brushtail possums are approximately 25 times larger than woodrats (3 kg vs. 120 g). Body mass is known to significantly influence the thermal tolerance of mammals, such that smaller mammals typically prefer warmer temperatures, have higher metabolic intensities, and are more metabolically responsive to shifts in ambient temperature compared to larger mammals (Gardner et al. 2011; Porter and Kearney 2009; Riesenfeld 1981; Scholander et al. 1950).

The vast majority of TDT studies have been conducted with rodents (Connors et al. 2017; Gordon et al. 2014; Gordon et al. 1988; Kaplanski and Ben-Zvi 1980; Keplinger et al. 1959; Kurnath and Dearing 2013; Kurnath et al. 2016; Settivari et al. 2008; Settivari et al. 2009; Windley and Shimada 2019). Rodents, and mice in particular, respond to toxic challenges by seeking cooler environments (Gordon et al. 2014). Typically this movement to the cool is accompanied by a dramatic decrease in body temperature (Gordon et al. 2014). A change to core body temperature results in changes to enzymatic activity in the sites of detoxification therein (Gordon et al. 2014; Gordon et al. 1988). For an endotherm without such fluctuations in core body temperature, the site of detoxification is unlikely to experience the changes in temperature that occurs in some rodents. This means that TDT could manifest differently in different species based on both body mass and unique physiological responses to thermoregulation. Ultimately, while mammals across a range of body sizes likely experience negative effects due to TDT, the physiological trade-off between thermoregulation and PSM detoxification may be more costly for smaller-bodied mammals ingesting PSMs. Further work could help to elucidate the intricacies of TDT and body mass in mammalian herbivores by comparing

effects of higher ambient temperatures across a range of masses in closely related species, and across a broader range of species.

Taken together, our results suggest that TDT has important implications for the ecology of mammalian herbivores, particularly in the context of global climate change. For example, the hepatic detoxification capacity of herbivores may be compromised during extreme weather events, such as prolonged heat waves, which are now occurring with higher frequency and intensity (Mazdiyasni and AghaKouchak 2015; Russo et al. 2014). Common brushtail possums and common ringtail possums are widely distributed across eastern Australia (Atlas of living Australia 2018), and have experienced higher than average ambient temperatures and more frequent extreme climatic events across their range (CSIRO and BOM 2015). Moreover, the PSM concentration in *Eucalyptus* leaves is predicted to increase with higher atmospheric carbon dioxide, although this is still an active area of research and there are many interacting factors (Bidart-Bouzat and Imeh-Nathaniel 2008; Craine et al. 2010; Gleadow et al. 2002; Lawler et al. 1996). Seasonal changes in ambient temperature are also likely to be accompanied by differences in the rate at which herbivores metabolise PSMs. Because feeding decisions in wild herbivores can be dictated by the metabolism of PSMs (e.g. Marsh et al. 2005), such changes could result in seasonal differences in foraging behaviour, beyond the effects of seasonal food availability (Beale et al. 2018; Dearing et al. 2005). Temperature-dependent changes in hepatic detoxification capacity may also have significant effects on ecosystem function, since plant-herbivore interactions can have significant flow on effects (Foley and Moore 2005). Further, relatively small changes in diet composition, such as a slight reduction in protein intake, can lead to reduced reproductive success (White 1983), which could potentially result in large changes at the population level. These “amplifier” effects mean that even small changes in diet should be considered significant (DeGabriel et al. 2009; White 1983).

Functional clearance time assays provided a practical, whole-animal, non-destructive technique for measuring liver function in mammals. We have improved upon previous methodology by utilizing a non-barbiturate drug that still acts as a proxy compound for ecologically relevant PSMs, while maintaining the “sleep time” aspect of the assay to assess functional clearance time. Other researchers seeking to expand knowledge of TDT and liver function to other herbivorous species, or to conduct non-destructive,

whole-organisms assays of liver function, would benefit by switching away from barbiturates to other anaesthetics like Alfaxalone. Understanding how ambient temperature and plant toxins interact to influence diet selection by herbivores will aid in efficient management of wild populations, as well as predicting potential ecosystem-wide responses to modern climate change. TDT is a phenomenon experienced by phylogenetically distinct mammalian herbivores, across a range of body sizes. It is likely that TDT could impact any endothermic species that must balance thermoregulation with hepatic detoxification of PSMs.

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This work was approved by the Australian National University Animal Experimentation

Ethics Committee and conforms to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

References

Atlas of living Australia (2018) www.ala.org.com.au. Accessed: February 2018

Barron DW, Dundee JW (1961) The recently introduced rapidly acting barbiturates; a review and critical appraisal in relation to thiopentone. Br J Anaesth 33:81-91

Beale PK, Marsh KJ, Foley WJ, Moore BD (2018) A hot lunch for herbivores: physiological effects of elevated temperatures on mammalian feeding ecology. Biol Rev Camb Philos Soc 93:674-692. doi: 10.1111/brv.12364

Berry MN, Clark DG, Grivell AR, Wallace PG (1985) The contribution of hepatic metabolism to diet-induced thermogenesis. Metabolism 34:141-147

Bidart-Bouzat MG, Imeh-Nathaniel A (2008) Global change effects on plant chemical defenses against insect herbivores. J Integr Plant Biol 50:1339-1354

Boyle RR, McLean S (2004) Constraint of feeding by chronic ingestion of 1,8-cineole in the brushtail possum (*Trichosurus vulpecula*). J Chem Ecol 30:757-775

Charkoudian N (2010) Mechanisms and modifiers of reflex induced cutaneous vasodilation and vasoconstriction in humans. J Appl Physiol 109:1221-1228. doi: 10.1152/japplphysiol.00298.2010

Chatelain M, Halpin CG, Rowe C (2013) Ambient temperature influences birds' decisions to eat toxic prey. Anim Behav 86:733-740

Connors PK, Malenke JR, Dearing MD (2017) Ambient temperature-mediated changes in hepatic gene expression of a mammalian herbivore (*Neotoma lepida*). Mol Ecol 26:4322-4338. doi: 10.1111/mec.14192

Craine JM, Elmore AJ, Olson KC, Tolleson D (2010) Climate change and cattle nutritional stress. Global Change Biol 16:2901-2911

CSIRO, BOM (2015) Climate change in Australia technical report. In: Whetton P (ed) Climate change in Australia information for Australia's natural resource management regions: technical report. CSIRO and Bureau of Meteorology, Australia

Dearing MD (2013) Temperature-dependent toxicity in mammals with implications for herbivores: a review. J Comp Physiol B 183:43-50

Dearing MD, Foley WJ, McLean S (2005) The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. Annu Rev Ecol Evol Syst 36:169-189

Dearing MD, Forbey JS, McLister JD, Santos L (2008) Ambient temperature influences diet selection and physiology of an herbivorous mammal, *Neotoma albigula*. Physiol Biochem Zool 81:891-897

Dearing MD, Skopec MM, Bastiani MJ (2006) Detoxification rates of wild herbivorous woodrats (*Neotoma*). Comp Biochem Physiol A Mol Integr Physiol 145:419-422. doi: 10.1016/j.cbpa.2006.07.016

DeGabriel JL, Moore BD, Foley WJ, Johnson CN (2009) The effects of plant defensive chemistry on nutrient availability predict reproductive success in a mammal. Ecology 90:711-719

Foley WJ, Moore BD (2005) Plant secondary metabolites and vertebrate herbivores – from physiological regulation to ecosystem function. Curr Opin Plant Biol 8:430-435. doi: 10.1016/j.pbi.2005.05.009

Freeland WJ, Janzen DH (1974) Strategies in herbivory by mammals: The role of plant secondary compounds. Am Nat 108:269-289

Gardner JL, Peters A, Kearney MR, Joseph L, Heinsohn R (2011) Declining body size: a third universal response to warming? Trends Ecol Evol 26:285-291. doi: 10.1016/j.tree.2011.03.005

Gleadow RM, Foley WJ, Woodrow IE (2002) Enhanced CO2 alters the relationship between photosynthesis and defence in cyanogenic *Eucalyptus cladocalyx* F. Muell. Plant, Cell Environ 21:12-22. doi: 10.1046/j.1365-3040.1998.00258.x

Gordon CJ, Johnstone AFM, Aydin C (2014) Thermal stress and toxicity. Compr Physiol 4:995–1016

Gordon CJ, Mohler FS, Watkinson WP, Rezvani AH (1988) Temperature regulation in laboratory mammals following acute toxic insult. Toxicology 53:161-178

Hales JR, Rowell LB, King RB (1979) Regional distribution of blood flow in awake heat-stressed baboons. Am J Physiol Heart Circ Physiol 237:H705-H712. doi: 10.1152/ajpheart.1979.237.6.H705

Hermsen E, Kerle A, Old JM (2016) Diet of an inland population of the common ringtail possum (*Pseudocheirus peregrinus*). Aust Mammal 38:130-134. doi: 10.1071/AM15008

Horowitz M (1998) Do cellular heat acclimation responses modulate central thermoregulatory activity? Physiology 13:218-225. doi: 10.1152/physiologyonline.1998.13.5.218

Kaplanski J, Ben-Zvi Z (1980) Effect of chronic heat exposure on *in-vitro* drug metabolism in the rat. Life Sci 26:639–642

Keplinger ML, Lanier GE, Deichmann WB (1959) Effects of environmental temperature on the acute toxicity of a number of compounds in rats. Toxicol Appl Pharmacol 1:156-161

Kohl KD, Weiss RB, Cox J, Dale C, Dearing MD (2014) Gut microbes of mammalian herbivores facilitate intake of plant toxins. Ecol Lett 17:1238-1246. doi: 10.1111/ele.12329

Krieger RI, Feeny PP, Wilkinson CF (1971) Detoxication enzymes in the guts of caterpillars: an evolutionary answer to plant defenses? Science 172:579-581. doi: 10.1126/science.172.3983.579

Kurnath P, Dearing MD (2013) Warmer ambient temperatures depress liver function in a mammalian herbivore. Biol Lett 9:20130562. doi: 10.1098/rsbl.2013.0562

Kurnath P, Merz ND, Dearing MD (2016) Ambient temperature influences tolerance to plant secondary compounds in a mammalian herbivore. Proc R Soc Lond B 283:20152387

Lautt WW (2007) Regulatory processes interacting to maintain hepatic blood flow constancy: Vascular compliance, hepatic arterial buffer response, hepatorenal reflex, liver regeneration, escape from vasoconstriction. Hepatol Res 37:891-903. doi: 10.1111/j.1872-034X.2007.00148.x

Lawler IR, Foley WJ, Eschler BM, Pass DM, Handasyde K (1998) Intraspecific variation in *Eucalyptus* secondary metabolites determines food intake by folivorous marsupials. Oecologia 116:160-169. doi: 10.1007/s004420050575

Lawler IR, Foley WJ, Woodrow IE, Cork SJ (1996) The effects of elevated CO2 atmospheres on the nutritional quality of *Eucalyptus* foliage and its interaction with soil nutrient and light availability. Oecologia 109:59-68. doi: 10.1007/s004420050058

Li Y, Feng Z, Swihart R, Bryant J, Huntly N (2006) Modeling the impact of plant toxicity on plant–herbivore dynamics. J Dyn Differ Equ 18:1021-1042. doi: 10.1007/s10884-006-9029-y

Marsh KJ, Foley WJ, Cowling A, Wallis IR (2003a) Differential susceptibility to Eucalyptus secondary compounds explains feeding by the common ringtail (*Pseudocheirus peregrinus*) and common brushtail possum (*Trichosurus vulpecula*). J Comp Physiol B 173:69-78. doi: 10.1007/s00360-002-0318-4

Marsh KJ, Moore BD, Wallis IR, Foley WJ (2014) Continuous monitoring of feeding by koalas highlights diurnal differences in tree preferences. Wildl Res 40:639-646

Marsh KJ, Wallis IR, Andrew RL, Foley WJ (2006a) The detoxification limitation hypothesis: where did it come from and where is it going? J Chem Ecol 32:1247-1266. doi: 10.1007/s10886-006-9082-3

Marsh KJ, Wallis IR, Foley WJ (2003b) The effect of inactivating tannins on the intake of Eucalyptus foliage by a specialist Eucalyptus folivore (*Pseudocheirus peregrinus*) and a generalist herbivore (*Trichosurus vulpecula*). Aust J Zool 51:31-42. doi: 10.1071/ZO02055

Marsh KJ, Wallis IR, Foley WJ (2005) Detoxification rates constrain feeding in common brushtail possums (*Trichosurus vulpecula*). Ecology 86:2946-2954. doi: 10.1890/05-0303

Marsh KJ, Wallis IR, McLean S, Sorensen JS, Foley WJ (2006b) Conflicting demands on detoxification pathways influence how common brushtail possums choose their diets. Ecology 87:2103-2112

Mazdiyasni O, AghaKouchak A (2015) Substantial increase in concurrent droughts and heatwaves in the United States. Proc Natl Acad Sci USA 112:11484-11489. doi: 10.1073/pnas.1422945112

McDowell A, McLeod BJ (2007) Physiology and pharmacology of the brushtail possum gastrointestinal tract: Relationship to the human gastrointestinal tract. Adv Drug Del Rev 59:1121-1132. doi: 10.1016/j.addr.2007.06.012

McLean S, Duncan AJ (2006) Pharmacological perspectives on the detoxification of plant secondary metabolites: implications for ingestive behavior of herbivores. J Chem Ecol 32:1213-1228. doi: 10.1007/s10886-006-9081-4

Miller ER, Folwer ME (2015) Fowler's Zoo and Wild Animal Medicine. Elsevier, St. Louis

Nespolo RF, Bacigalupe LD, Sabat P, Bozinovic F (2002) Interplay among energy metabolism, organ mass and digestive enzyme activity in the mouse-opossum *Thylamys elegans*: the role of thermal acclimation. J Exp Biol 205:2697-2703

Ohnhaus EE, Tilvis R (1976) Liver blood flow, metabolic heat production and body temperature before, during and after phenobarbitone administration. Acta Hepatogastroenterol (Stuttg) 23:404-408

Pahl L (1987a) Feeding-behavior and diet of the common ringtail possum, *Pseudocheirus-Peregrinus*, in *Eucalyptus* woodlands and *Leptospermum* thickets in southern Victoria. Aust J Zool 35:487-506. doi: 10.1071/ZO9870487

Pahl L (1987b) Survival, age-determination and population age structure of the common ringtail possum, *Pseudocheirus Peregrinus*, in a *Eucalyptus* woodland

and a *Leptospermum* thicket in southern Victoria. Aust J Zool 35:625-639. doi: 10.1071/ZO9870625

Pass GJ, McLean S, Stupans I (1999) Induction of xenobiotic metabolising enzymes in the common brushtail possum, *Trichosurus vulpecula*, by *Eucalyptus* terpenes. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 124:239-246

Pass GJ, McLean S, Stupans I, Davies N (2001) Microsomal metabolism of the terpene 1,8-cineole in the common brushtail possum (*Trichosurus vulpecula*), koala (*Phascolarctos cinereus*), rat and human. Xenobiotica 31:205-221. doi: 10.1080/00498250110043535

Périard JD, Travers GJS, Racinais S, Sawka MN (2016) Cardiovascular adaptations supporting human exercise-heat acclimation. Auton Neurosci 196:52-62. doi: 10.1016/j.autneu.2016.02.002

Porter WP, Kearney M (2009) Size, shape, and the thermal niche of endotherms. Proc Natl Acad Sci USA 106:19666-19672. doi: 10.1073/pnas.0907321106

Provenza FD, Villalba JJ, Dziba LE, Atwood SB, Banner RE (2003) Linking herbivore experience, varied diets, and plant biochemical diversity. Small Ruminant. Res 49:257-274. doi: 10.1016/S0921-4488(03)00143-3

Riek A, Geiser F (2013) Allometry of thermal variables in mammals: consequences of body size and phylogeny. Biol Rev Camb Philos Soc 88:564-572. doi: 10.1111/brv.12016

Riesenfeld A (1981) The role of body mass in thermoregulation. Am. J. Phys. Anthropol. 55:95-99. doi: 10.1002/ajpa.1330550113

Russo S, Dosio A, Graversen RG, Sillmann J, Carrao H, Dunbar MB, Singleton A, Montagna P, Barbola P, Vogt JV (2014) Magnitude of extreme heat waves in present climate and their projection in a warming world. J Geophys Res Atmos 119:12,500-512,512. doi: 10.1002/2014JD022098

Sasaki N (1994) Effects of furazolidone on duration of righting reflex loss induced with hexobarbital and zoxazolamine in the rat. J Vet Med Sci 56:667-670. doi: 10.1292/jvms.56.667

Scholander PF, Hock R, Walters V, Johnson F, Irving L (1950) Heat regulation in some arctic and tropical mammals and birds. Biol Bull 99:237-258. doi: 10.2307/1538741

Settivari RS, Evans TJ, Eichen PA, Rottinghaus GE, Spiers DE (2008) Short- and long-term responses to fescue toxicosis at different ambient temperatures. J Therm Biol 33:213-222

Settivari RS, Evans TJ, Yarru LP, Eichen PA, Sutovsky P, Rottinghaus GE, Antoniou E, Spiers DE (2009) Effects of short-term heat stress on endophytic ergot alkaloid-induced alterations in rat hepatic gene expression. J Anim Sci 87:3142-3155

Speakman JR, Król E (2010) Maximal heat dissipation capacity and hyperthermia risk: neglected key factors in the ecology of endotherms. J Anim Ecol 79:726-746

Toloza EM, Lam M, Diamond J (1991) Nutrient extraction by cold-exposed mice: a test of digestive safety margins. Am J Physiol Gastrointest Liver Physiol 261:G608-G620. doi: 10.1152/ajpgi.1991.261.4.G608

Torregrossa A-M, Dearing MD (2009) Nutritional toxicology of mammals: regulated intake of plant secondary compounds. Funct Ecol 23:48-56. doi: 10.1111/j.1365-2435.2008.01523.x

Twigg LE, Martin GR, Eastman AF, King DR, Kirkpatrick WE (2003) Sensitivity of some Australian animals to sodium fluoroacetate (1080): additional species and populations, and some ecological considerations. Aust J Zool 51:515-531. doi: 10.1071/ZO03040

Wang Z, Ying Z, Bosy-Westphal A, Zhang J, Schautz B, Later W, Heymsfield SB, Müller MJ (2010) Specific metabolic rates of major organs and tissues across adulthood: Evaluation by mechanistic model of resting energy expenditure. Am J Clin Nutr 92:1369-1377

Warne LN, Beths T, Whittem T, Carter JE, Bauquier SH (2015) A review of the pharmacology and clinical application of alfaxalone in cats. T Vet J 203:141-148. doi: 10.1016/j.tvjl.2014.12.011

White RG (1983) Foraging patterns and their multiplier effects on productivity of northern ungulates. Oikos 40:377-384. doi: 10.2307/3544310

Windley HR, Shimada T (2019) Cold temperature improves tannin tolerance in a granivorous rodent. J Anim Ecol 0. doi: 10.1111/1365-2656.13119

Winter J (1980) Tooth wear as an age index in a population of the brush-tailed possum, *Trichosurus vulpecula* (Kerr). Wildl Res 7:359-363. doi: 10.1071/WR9800359

Chapter 3

Changes in ambient temperature can be as important as plant secondary metabolites in limiting feeding in mammalian herbivore

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Abstract

Until recently, the tolerance of herbivores to PSMs was considered an intrinsic feature of their physiology. Here we show that the consumption of PSM-containing foods is strongly influenced by exposure to different ambient temperatures (T_a) in common brushtail possums (*Trichosurus vulpecula*) and common ringtail possums (*Pseudocheirus peregrinus*). We measured the metabolic rates of ringtail possums at different T_a's, so that we could choose temperatures for later experiments. Metabolic rate in ringtail possums between 4-35°C was best described by a continuous curve rather than the traditional Scholander broken stick model of thermoneutrality. We chose three temperatures to study the effect of T_a on feeding; 10 °C (below the thermoneutral zone), 18 °C (approximately lower critical temperature) and 26 °C (approximately upper critical temperature). For both possum species, the amount eaten of food containing high concentrations of PSMs depended on T_a and on the length of time that they were exposed to that temperature. The animals ate less PSM-rich food when exposed to high T_a than when exposed to a low T_a, if they were exposed to that temperature for one week. When their exposure was limited to one day there was no effect of temperature on the intake of PSM-rich food by ringtail possums, and the effect of temperature was reduced in brushtail possums. Our results are consistent with the hypothesis of temperature dependent toxicity whereby hepatic enzymes responsible for metabolism of absorbed PSMs are downregulated when the ability to dissipate heat is limited. It is vital that T_a effects on nutrition are considered when seeking to understand the impact of climatic shifts on ecosystems.

Key words

Temperature-dependent toxicity, thermoregulation, herbivory, plant secondary metabolite, ambient temperature, nutritional ecology

Introduction

Interactions between plants and herbivores are a fundamental component of ecosystem dynamics. With predictions of warmer average temperatures, greater temperature fluctuations, and more heat waves (Intergovernmental Panel on Climate Change 2007), global climate change is likely to affect plants and herbivores in a variety of ways. However, there has been surprisingly little focus on the direct effects of higher ambient temperatures (T_a) on the nutritional decisions of wild mammalian herbivores. For example, at the simplest level, we know that animals in production systems consume less food at high T_a (e.g. Bernabucci et al. 1999; Ominski et al. 2002; Renaudeau et al. 2002). This is not just due to reduced energy requirements, but also because of limits to dissipating additional heat from digestion and metabolism (Dale and Fuller 1979; Herd et al. 2004; Renaudeau et al. 2012).

Increases in T_a may be particularly challenging for wild vertebrate herbivores consuming diets rich in plant secondary metabolites (PSMs). These animals may have to limit how much food they eat at high T_a because of interactions between thermoregulation and metabolism of PSMs (Beale et al. 2017). In addition, there may be complex interactions between nutrient composition, PSM concentration, and temperature (Camp et al. 2018). Of particular concern is that many xenobiotics become more toxic when T_a is high (Chapter 2, Keplinger et al. 1959; Kurnath et al. 2016; Oliver and King 1983). This is known as temperature-dependent toxicity (TDT; Dearing 2013).

Dearing (2013) hypothesised that the effect of PSMs on the dietary intake of mammalian herbivores would be influenced by their thermal response, such that intake would be highest around the lower critical temperature (LCT) of the thermoneutral zone (TNZ). The TNZ is the range of ambient temperatures over which basal metabolic rate is lowest and relatively constant in endotherms. The traditional Scholander model of temperature and metabolism has the TNZ being demarcated by an upper critical temperature (UCT) and a LCT, beyond which metabolic rate is increased. Dearing's (2013) hypothesis was based on findings of three toxicological studies with rodents. One used rat liver isolates *in vitro* (Kaplanski and Ben-Zvi 1980), one involved acute

intra-peritoneal injection of toxicants and measurement of LD50 (Keplinger et al. 1959), while the last involved oral administration of toxicants (Settivari et al. 2008).

A number of toxicants in Keplinger (1959) show the pattern predicted by Dearing (2013), however they are largely central nervous system depressants. It is possible that at cooler temperatures these toxicants prevent central initiation of thermoregulatory mechanisms, physiological and/or behavioural, resulting in body temperature fluctuations which impact hepatic metabolism, or simply render the rats more susceptible to the cold (Gordon et al. 1988). Aside from a handful that directly cause hypothermia, most other compounds tested by Keplinger (1959) show patterns of toxicity more consistent with the results of our previous study in possums, whereby the rate of metabolism of an anaesthetic agent, Alfaxalone, increased with increasing temperature (Chapter 2). Other features of Keplinger (1959) that make comparison difficult are that rats were exposed to temperature treatments for just 45 mins, and the intraperitoneal route of administration precludes any temperature effects taking place in the gut. What we can glean from Keplinger (1959) is that, while temperature is important for toxicant metabolism, the direct actions of the compound in question, if known, must be considered when interpreting results, and importantly the absorption, diffusion, distribution, or metabolism of a compound could all be altered by T_a. Therefore, to best capture the impact of T_a on ingested PSMs, ideally, test compounds are orally administered.

In a study more closely resembling herbivores ingesting PSMs, rats were offered seed diets with and without endophyte infestation (Settivari et al. 2008). However, fescue toxicosis is a problematic poster child of TDT as the toxin directly interferes with heat loss mechanisms by preventing peripheral vasodilation. It is unsurprising, therefore, that there are temperature-dependent effects on intake and toxicity. What is interesting is the timing of such effects. Settivari (2008) showed that there were short term changes in response to the endophyte infested seed at 21°C (well below the TNZ of rats) that diminished over time, while at 31°C some effects were enhanced with longer exposure. While the physiological processes that underpin TDT are likely multifaceted, downregulation of hepatic metabolism appears to be a key component (Beale et al. 2017; Connors et al. 2017; Dearing 2013; Kurnath and Dearing 2013). In common brushtail possums (*Trichosurus vulpecula*) and common ringtail possums

(*Pseudocheirus peregrinus*), week-long exposure to seemingly mild increases in T_a's decreased hepatic detoxification rate of the common anesthetic alfaxan, whereas overnight exposure to temperature adjustments were not long enough to induce these effects. Using similar techniques, the hepatic detoxification rate of hexobarbital in woodrats was also shown to be reduced by exposure to warm T_a's (Kurnath and Dearing 2013). Although in a previous study, overall resting metabolic rate in woodrats was decreased by ingestion of juniper (Boyle and Dearing 2003). Since herbivores closely regulate their intake of PSMs (Marsh et al. 2005; Torregrossa and Dearing 2009), changing limits to detoxification could cause them to markedly alter feeding (Boyle and McLean 2004; Dearing et al. 2008; Marsh et al. 2006a; Marsh et al. 2005).

While it has been well established that PSMs are an important influence of feeding decisions in many herbivores (Dearing et al. 2005; Foley and Moore 2005; Forbey et al. 2013; Marsh et al. 2006a), until recently, their tolerance for PSMs has been considered a purely intrinsic feature of their physiology. If the tolerance of herbivores to PSMs changes with an extrinsic abiotic factor such as temperature exposure, then it is imperative we consider this dynamic relationship when seeking to understand how herbivores will respond to changes in climate. For example, in an examination of future climate change impacts on the greater glider (*Petauroides volans*; a folivorous gliding marsupial) mechanistic niche modelling was used to integrate nutrition with changes to abiotic factors, to predict future species distributions (Kearney et al. 2010). However, despite PSMs playing a major role in diet selection by greater gliders (Youngentob et al. 2011), they were not included in the model. There is opportunity to adapt these models to include PSMs, but also to consider interactions between metabolism of PSMs and thermoregulation (Forbey et al. 2018; Kearney et al. 2012; Kearney et al. 2010). If T_a and PSM tolerance are integrated, together with nutrient availability, physiologically based models and mechanistic niche models could be used more effectively (Forbey et al. 2018). However, these models need to be informed by experimental evidence specific to the system in question.

To better understand these relationships, we investigated the effect of variation in T_a on feeding in two well-studied marsupial herbivores, the common brushtail possum and common ringtail possum. Both of these species typically eat diets rich in PSMs. Some of these PSMs have been shown to limit intake (e.g.Marsh et al. 2005; Marsh et al. 2006b),

but the effects of T_a on their feeding ecology have not been examined. We first attempted to characterise the TNZ of both species to determine appropriate temperatures for feeding experiments. We were unable to determine the TNZ of brushtail possums, however, due to individuals developing stress colitis following respirometry. Nevertheless, there are some previous data on their thermal tolerance in the literature (Dawson and Olson 1988). We then tested whether T_a affected the voluntary food intake of brushtail and ringtail possums exposed to different T_a for different time periods. Brushtail possums were offered an artificial diet with or without the PSM, flavanone. Flavanone is a representative of a class of PSMs present in the foliage of many eucalypt species (Marsh et al. 2019; Saraf et al. 2017), and is known to deter brushtail possums from feeding (Marsh et al. 2015). Ringtail possums are difficult to maintain on an artificial diet, so they were offered *Eucalyptus melliodora* leaves that naturally contained either a low or high concentration of another PSM, sideroxylonal, a formylated phloroglucinol compound that strongly deters feeding by ringtail possums (Lawler et al. 2000; Wiggins et al. 2006).

We hypothesised that intake of the basal diet by brushtail possums and the low-sideroxylonal leaves by ringtail possums would decrease with increasing T_a following a week-long exposure to a particular temperature (Figure 1). This is because, for diets containing significant concentrations of PSMs, intake is determined by the rate of metabolism of PSMs. In our previous study, both brushtail and ringtail possums had a reduced rate of hepatic metabolism following a week-long exposure to warmer T_a's (Chapter 2). In contrast, when exposed to the same temperatures for up to 17 h overnight, there was no difference in the rate of hepatic metabolism. Thus, we hypothesised that there would be no effect of temperature on the intake of PSM-containing diets, when possums were exposed to different temperatures overnight (Figure 1).

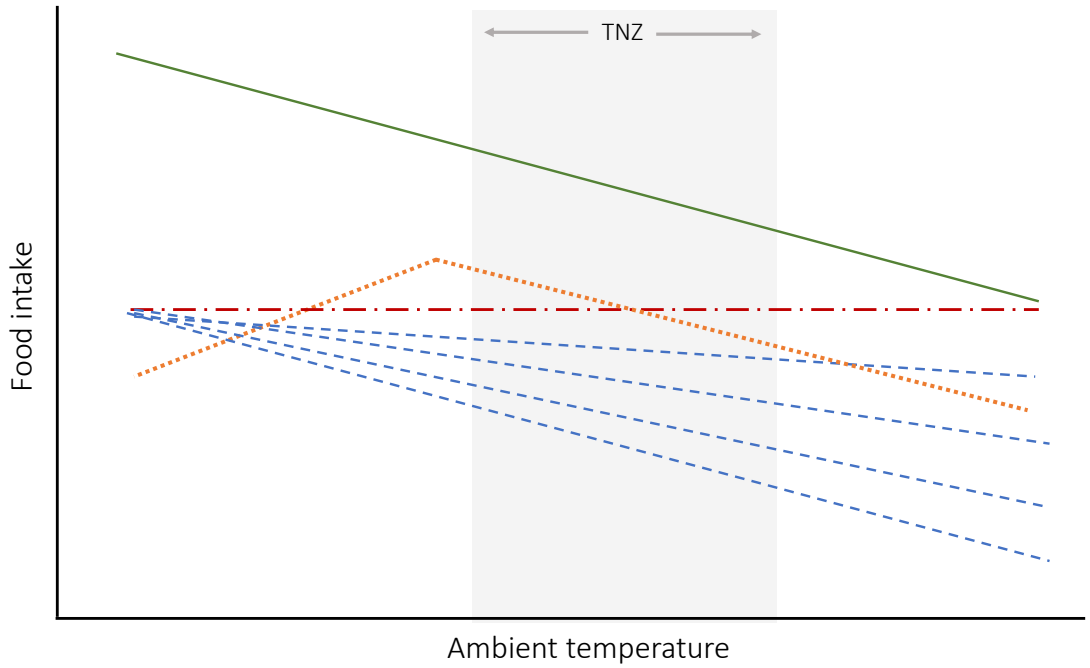

Fig. 1 *Predicted food intake at a range of ambient temperatures. Expected food intake of a PSM-free diet is indicated by the solid green line. All other lines represent intake of diets containing PSMs. Note that predicted food intake does not reflect any increase in energy requirements above the UCT even for a PSM-free diet. The red dot-dash line indicates hypothesised intake if there is no effect of ambient temperature on PSM tolerance. If intake of a PSM containing diet is reduced below preferred intake to a level determined by metabolism of the PSM, and metabolism of the PSM doesn't change with temperature, intake should be constant across different temperatures. The orange dotted line is adapted from Dearing (2013) and represents the author's prediction that tolerance to PSMs may be highest just below the lower critical temperature of the thermoneutral zone (TNZ), indicated by grey shading. The blue dashed lines indicate intake patterns if increasing ambient temperature reduces tolerance to PSMs linearly across temperatures. Multiple patterns of TDT are possible such that intake of PSM-containing diets changes with ambient temperature all at levels below the intake of a PSM free diet (Keplinger et al. 1959)*

Methods

Husbandry of common ringtail possums

Twelve common ringtail possums (five non-lactating females and seven males; mean ± se = 743 ± 24 g) were caught by hand or from nest boxes on Black Mountain, Australian Capital Territory, at approximately 35.2667°S, 149.1000°E and 786m ASL in autumn and early winter 2015. Ringtail possums were weighed weekly and at the start and finish of each respirometry experiment. Changes in mass were calculated relative to the weight recorded for the previous week. During respirometry, possums were housed individually in large 6 x 3 x 3 m (l x w x h) aviaries at ambient temperature (10-20^{o}C) and light. For the later feeding experiments the animals were housed individually in smaller aviaries (66 x 65 x 92 cm) in three temperature controlled rooms (10^{o}C, 18^{o}C and 26^{o}C) on a 12:12 h light:dark cycle with a 15 W red light during the dark cycle. The intensity of light was changed over a 40 min period to simulate dawn and dusk. All animals were provided with nest boxes (15 x 15 x 20 cm) with one side formed from wire mesh to minimise temperature buffering.

Ringtail possums were fed *Eucalyptus* foliage *ad libitum* (primarily *E. rossii*) daily at 1630 h, and food was removed at 0900 h the following morning. The stems were placed in containers of water to prevent drying. A control bunch of leaves was used to monitor changes in mass, but these changes were always small (average of 0.5 g), so no correction was made during calculations of foliage intake. Dry matter intake (DMI) was determined by drying a subsample of leaves at 50 ^{o}C from the control bunch and multiplying this by the mass of fresh leaves consumed. Water was available *ad libitum*.

Husbandry of common brushtail possums

Twelve adult male brushtail possums were caught in cage traps on The Australian National University campus, Canberra, in March 2015. These animals were used for two feeding experiments. Brushtail possums were housed in aviaries (90 x 70 x 152cm) with an open three-sided nest platform (18 x 18 x 34cm). This was to prevent temperature buffering by the nest box. The aviaries were inside constant temperature rooms identical to those described above. We measured the body mass of brushtail possums

weekly, and changes in mass were calculated relative to the weight recorded the previous week.

Brushtail possums were initially fed a mixture of leaves and fresh fruit. Fresh water was always available. Over a period of five weeks, brushtail possums were transitioned to a basal diet of mashed fruit and cereals. The diet was prepared fresh daily. The basal diet consisted of 42.5% chopped apple, 28% chopped banana, 10% chopped carrot, 6% ground rice hulls, 6% rolled oats, 4% ground lucerne chaff, 1.67% acid casein, 1.25% vegetable oil, 0.3% NaCl, 0.25% dicalcium phosphate, and 0.03% vitamin and mineral mix (Nutrimol, Vitagran: Multicrop Pty Ltd, Scoresby, Australia), all on a wet matter basis. The basal diet contained approximately 13% crude protein, 29% neutral detergent fibre and 7% crude fat. The total nitrogen concentration of the diet was determined by combustion using a LECO TruSpec® CN analyser. Fat was analysed using accelerated solvent extraction (Schäfer 1998). Neutral detergent fibre (NDF) was determined using fibre bag extraction for the ANKOM technology A2000 fibre analyser (Vogel 1999). Food was offered at 1630 h and refusals collected at 0900 h daily throughout all experiments. The dry matter content of the offered food diet was measured daily. Dry matter intake was then determined by drying all leftover food, and subtracting this from the amount of dry matter offered:

$$INTAKE_{DRY} = (CONTROL_{DRY} / CONTROL_{WET}) * OFFER_{WET} - UNEATEN_{DRY}$$

Respirometry

We originally planned to measure the metabolic rate at different T_a for both brushtail and ringtail possums using respirometry. However, half of the brushtail possums suffered a stress colitis following their first day of measurements, and, despite attempts to acclimate them to the procedure, the colitis returned. Therefore, only data for ringtail possums is described.

Resting metabolism and body temperature in common ringtail possums were measured using positive pressure flow through respirometry. Fresh air from outside the building was pumped through a silica gel desiccant at a rate of 2000 $mL.min^{-1}$ using a mass flow regulator, and was heated or cooled to a set temperature using a Sable Systems PELT-5 Peltier Effect Temperature Controller and Peltier Effect unit (Sable Systems

International, NV, USA). The air was then directed via copper coils into the airtight metabolism chamber (300 x 300 x 300 mm), which contained a dry branch as a perch, and a wire mesh floor elevating the possum from a layer of vegetable oil. Faeces and urine produced by the animal during measurements were collected below the layer of oil so that they did not interfere with measurements. The chamber and copper coils were all held within a larger insulated temperature-controlled cabinet. All measurements were taken within the daylight hours, between 0800 h and 1730 h, which is the resting phase for this species. The animals were not fasted prior to the measurements and even though feeding would have ceased at about 0400 h (personal observations), undoubtedly food would have been in the digestive system when the measurements began.

A calibrated Type T 32-gauge thermocouple wire was inserted approximately 5 cm into the rectum of each possum and taped to the tail. A second thermocouple wire was taped to the inside of the metabolism chamber to monitor chamber temperature. These thermocouples were attached to a thermocouple reader (TC-1000; Sable Systems International, NV, USA), and a UI-2 system interface and collected onto a personal computer into the Warthog Lab Helper X Software (WartHog Systems, University of California). After being placed into the chamber, the possum was left until the respiratory rate and pattern were regular, the O_2 and CO_2 trace had plateaued, and the chamber had stabilised at the predetermined temperature. This normally took about one hour.

Before and after the measurement of metabolic rate, the oxygen, carbon dioxide, and water vapour in atmospheric air was analysed as a baseline. Once the respiratory rate had stabilised for each temperature, 10 samples of respiratory rate and pattern were measured using whole body plethysmography, and captured into Warthog Lab Helper X Software over a 20 min period before switching back to baseline. Airflow, oxygen, carbon dioxide, and water vapour density were measured continuously using the Sable Systems FoxBox Respirometry System (Sable Systems International, NV, USA), which subsampled 300 $mL.min^{-1}$ of excurrent air from the chamber via a manifold and three-way tap system. Using the FoxBox, this subsampled air travelled from the chamber sequentially through a humidity sensor, a desiccant of silica gel and 'Drierite', a CO_2 sensor, a second 'Drierite' desiccant and 'Ascarite' CO_2 scrubber, followed by a differential oxygen analyzer. This data was captured using Expedata software. The dessicants and Ascarite were changed between each temperature sampling period, before baseline sampling. Output from all

sensors was acquired every 8 seconds into a personal computer using a 12-bit analogue to digital converter.

Measurements of gas exchange were taken at six different temperatures (three temperatures on each of two separate days in the chamber) for each ringtail possum. The temperatures were stratified so that possums experienced one low (4-15^{o}C), one medium (15-25^{o}C) and one high temperature (25-35^{o}C) on each day in the chamber. In this way, the maximum number of temperatures within the 4-35^{o}C range was covered, while allocation of temperatures to individuals within the categories was random. No animal experienced the same temperature twice, and at least two animals were exposed to every temperature. Only one individual was used each day and there was at least a week between the two days that each individual was in the metabolic chamber.

Resting metabolic rate (RMR) calculations were made using Warthog lab analyst software. Flow was taken as actual flow for each time-period and air was assumed to be dry. There were in total 75 data points (individual-temperature combinations) after faulty files and or experiments where equipment failure occurred were excluded.

In R studio, the 1st, 2nd, 3rd, and 4th order polynomial regressions were calculated on RMR and body temperature data with RMR or body temperature as the dependent variable and the temperature of the metabolic chamber as the independent variable. These were compared using AIC model selection criteria. Using the R package "segmented" the models were then assessed for potential breakpoints. This method tests for piecewise relationships in regression models using an iterative approach (Muggeo 2003).

The effect of long exposure to constant ambient temperatures on the intake of eucalypt leaves by common ringtail possums

Although the nature of the data from the respirometry experiment made it difficult to be precise, 18 °C was estimated to be close to or just below the LCT of ringtail possums, and 26 °C was close to the UCT (see results). We therefore chose to use temperatures of 10 °C, 18 °C and 26 °C for all further experiments. In the period before feeding experiments began, ringtail possums were offered leaves from the two experimental *Eucalyptus melliodora* trees to reduce any effects of novelty during the experiment. The

two trees differed in their concentration of the PSM, sideroxylonal (a combination of the three isomers Sideroxylonal A, B and C), which is known to act as a feeding deterrent (Lawler et al 2000; Jensen et al 2014). One tree had a higher concentration of sideroxylonal (2.2 $mg.g^{-1}$ DM), while the other had a low concentration (0.3 $mg.g^{-1}$ DM). A randomised crossover design was used to allocate 12 possums to a starting temperature of either 10°C, 18°C or 26°C. Animals were exposed to these temperatures for seven nights, followed by two experimental nights. Possums were offered *E. rossii* during the first seven days, and were offered leaves from one of the two *E. melliodora trees* on day eight, with the leaves from the second tree being offered on day nine. The experiment was repeated until the intake of leaves from both trees had been measured for every ringtail possum at all three temperatures.

The effect of short exposure to constant ambient temperatures on the intake of eucalypt leaves by common ringtail possums

All ringtail possums were held at 18 °C for a week preceding the experiment, and were offered leaves of *E. rossii*. On the eighth day, the 12 animals were divided into high (26 °C) and low (10 °C) temperature treatment groups at 1600 h. At 1630 h, half of the animals in each temperature treatment were offered the high-sideroxylonal *E. melliodora* leaves, and half were offered the low-sideroxylonal *E. melliodora* leaves. Food was removed at 0900 h the following morning, and at 1600 h, all possums were transferred back to 18 °C and offered leaves from the second *E. melliodora* tree. The experiment was repeated over the next two nights so that each possum received both diets at one of the experimental temperatures and at 18 °C. In other words, six possums were exposed to the high temperature treatment, and six the low, with the order of leaf treatments randomised for each possum in a balanced design.

The effect of long exposure to constant ambient temperatures on the intake of flavanone by common brushtail possums

The long temperature exposure experiment with brushtail possums followed the same experimental design as that with ringtail possums. Thus, a randomised crossover design was used to allocate 12 animals to a starting temperature of either 10°C, 18°C or 26°C. Animals were exposed to these temperatures for seven nights followed by two experimental nights. Animals were offered the basal diet during the first seven days,

and were offered either the basal diet or the basal diet containing 1.25% DM flavanone (a concentration known to deter feeding; Marsh et al. 2015) on day eight. The alternative diet was offered on day nine. As with the ringtail possums, the experiment was repeated until the food intake of the basal diet and that containing flavanone had been measured for every brushtail possum at all three temperatures.

The effect of short exposure to constant ambient temperatures on the intake of flavanone by common brushtail possums

The experimental design for studying feeding following a short temperature exposure in brushtail possums again followed the design used for ringtail possums. All possums were housed at 18°C for one week prior to the experiment, and were fed the basal diet. Possums were then randomly allocated into groups that alternated between either 18°C and 10°C or between 18°C and 26°C (six possums in each group). Possums were held at their allocated temperature at 1600 h and food intake of either the basal diet or the basal diet containing 1.25% DM flavanone was measured between 1630 h and 0900 h the next day. Diet and temperature treatments were swapped until the food intake of six possums had been measured at 10°C and 18°C for both diets, and likewise for six possums at 18°C and 26°C.

Statistical analysis of feeding experiments

All feeding experiments were analysed separately using generalised mixed models (packages; lme4, MuMIn, lmerTest, plyr) in R studio. We included temperature and diet as fixed effects, and animal identity and experimental period as the random effects, with dry matter intake as the dependent variable. An ANOVA was used to determine whether the fixed effects and the interaction term between fixed effects had a significant effect on the model.

Results

Resting metabolic rate and body temperature in ringtail possums

A second order polynomial best explained the relationship between resting metabolic rate (RMR) and T_a (multiple R^2 = 0.6812, rse = 0.3117, $p < 0.001$; Figure 2a). A single

breakpoint in the curve was estimated at 19.3°C (se = 1.378) (Figure 2a). However, this breakpoint was not significant (p=0.454), and when AIC model selection criteria were used to select the best model to fit the data, the original second order polynomial model with no breakpoint was selected.

The relationship between body temperature at the end of each respirometry measurement period and chamber temperature for that period was best explained by a third order polynomial (multiple R^2 = 0.1759, rse = 0.4111, p = 0.005). A single breakpoint in the curve was estimated at 26.3°C (se = 0.929) (Figure 2b). There was a trend for this breakpoint to be significant (p=0.09), but when AIC model selection criteria was used to select the best model to fit the data, the original third order polynomial model with no breakpoint was selected.

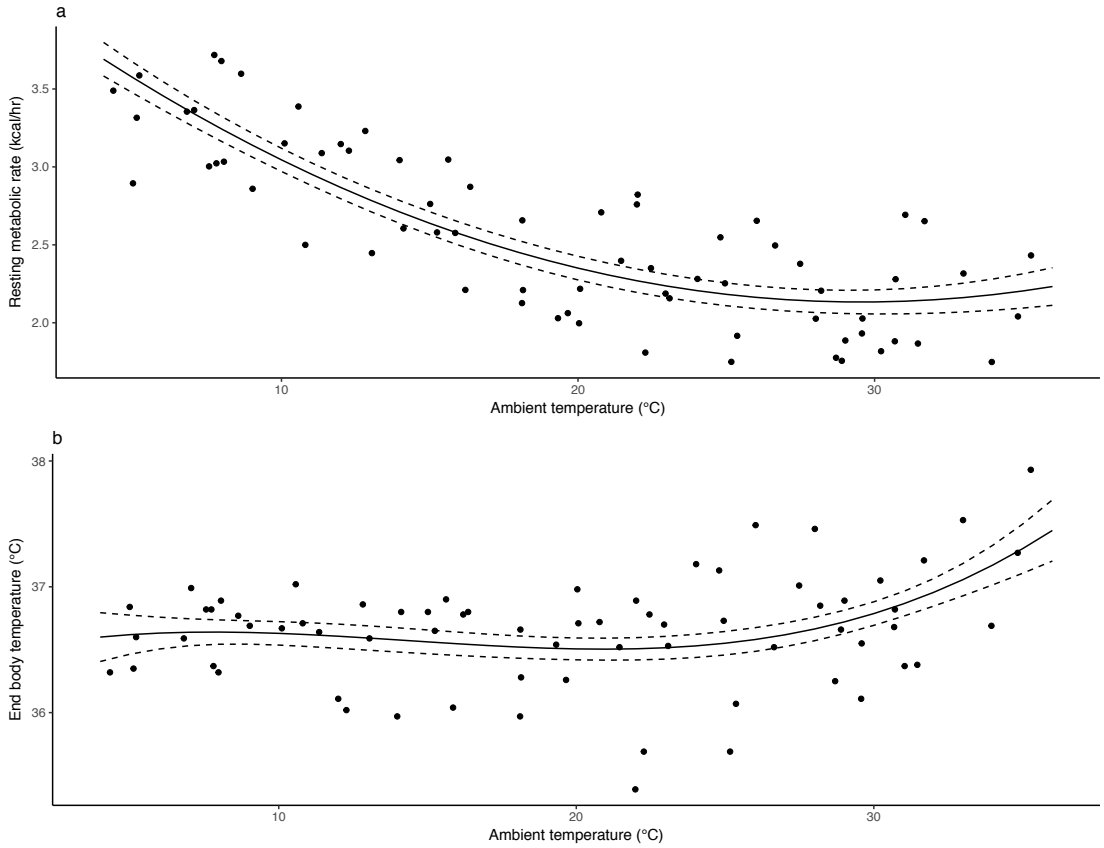

Fig. 2 The relationship between ambient temperature and a) resting metabolic rate, and b) body temperature at temperatures between 4°C and 35°C. The solid lines are the best fit from the statistical model, with standard error (dashed lines), points are individual measurements

The effect of ambient temperature on the intake of eucalypt leaves by common ringtail possums

In the short exposure experiment, possums ate less of the tree with a higher sideroxylonal concentration than of the low sideroxylonal tree ($F(1) = 3.33$, $P = 0.079$) and there was a negative relationship between food intake and temperature ($F(2) = 3.48$, $P = 0.043$), There was a significant interaction between temperature and diet ($F(2) = 5.21$, $P = 0.012$), such that ringtail possums ate significantly less of the low-sideroxylonal leaves following overnight exposure to 26°C compared to either 10°C or 18°C (Figure 3) while there was no significant difference in intake of high sideroxylonal leaf in any of the temperature treatments.

Following a week of exposure to each temperature, possums ate less of the tree with higher sideroxylonal concentration than of the low sideroxylonal tree ($F(1) = 54.68$, $P < 0.001$) and there was a negative relationship between food intake and temperature ($F(2) = 14.59$, $P < 0.001$). The interaction term between diet and temperature was not significant, showing that ringtail possums responded to T_a in a similar manner for both diets (Figure 3b).

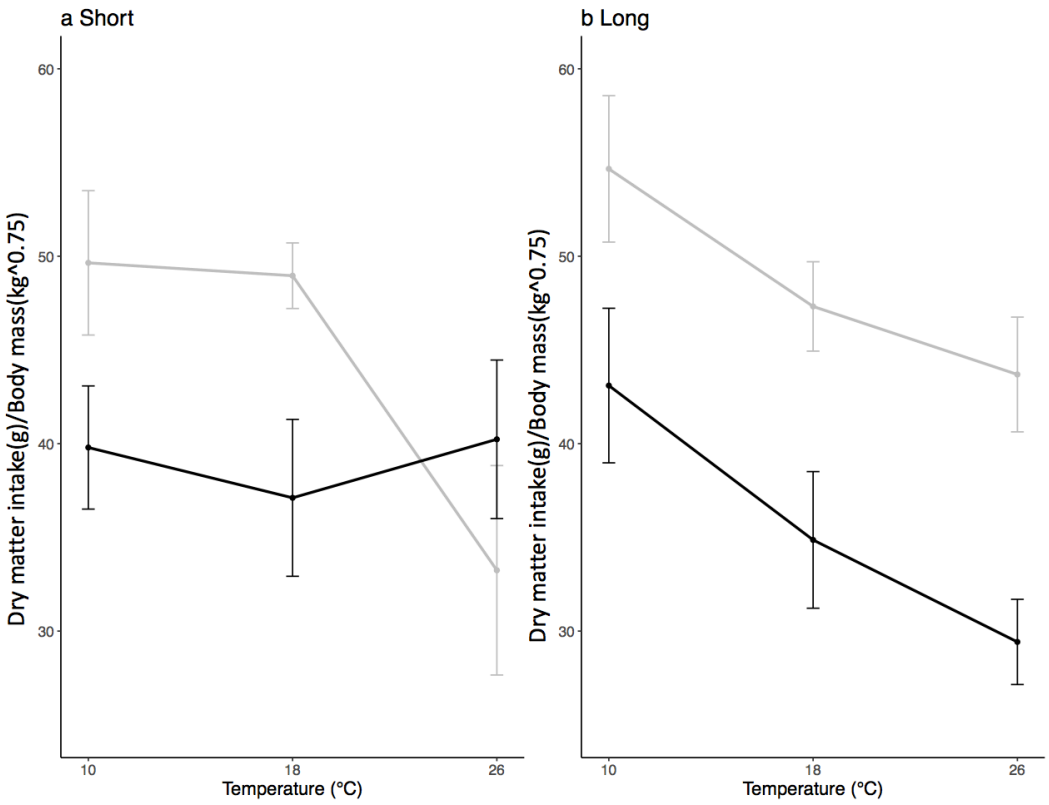

Fig. 3 The effect of a) short exposure, and b) long exposure to three ambient temperatures on the food intake (mean DMI ± se) of ringtail possums offered E. melliodora *foliage with a high (black) or low (grey) concentration of sideroxylonal*

The effect of ambient temperature on feeding by common brushtail possums

In the short exposure experiment, possums ate less of the diet containing flavanone than of the basal diet ((F(1) =16.83, P < 0.001), and there was a negative relationship between food intake and temperature (F(2) = 6.08, P = 0.005). The interaction terms were not significant, indicating that brushtail possums responded to the T_a in a similar manner on both diets (Figure 4a).

Following a week of exposure to different ambient temperatures, possums again ate less of the diet containing flavanone than of the basal diet (F(1) = 80.66, P < 0.001), and there was a negative relationship between food intake and temperature (F(2) = 6.08, P < 0.001). The interaction terms were not significant, indicating that brushtail possums responded to the T_a in a similar manner on both diets (Figure 4b).

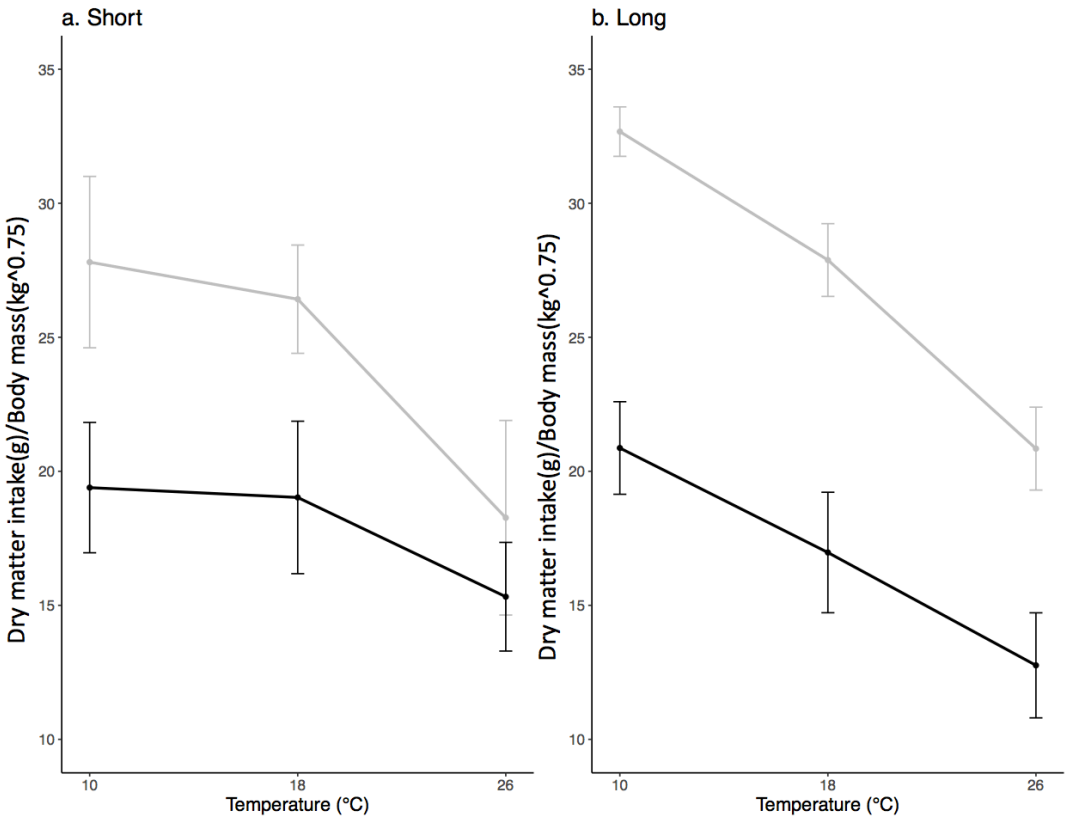

Fig. 4 *The effect of a) short exposure, and b) long exposure to three ambient temperatures on the food intake (mean ± se) of brushtail possums offered a diet with (black) or without (grey) flavanone*

Changes in body mass during feeding experiments

Relative to their mass the previous week, ringtails, on average, gained 9.1% of their body mass (se = 3.2) after one week at 10°C, lost 4.3% (se = 1.2) at 18°C, and lost 6.6% (se = 2.9) at 26°C. Brushtails, on average, gained 1.4% of their body mass (se= 0.7) after one week at 10°C, gained 0.5% (se = 0.8) at 18°C, and lost 2.3% (se = 0.7) at 26°C.

Discussion

Food intake was affected by ecologically relevant T_a in both brushtail and ringtail possums. The magnitude of the effects depended on both the amount of time that possums were exposed to a particular T_a and the presence of PSMs, but were surprisingly large given the relatively mild differences in T_a, and the relatively short

timescales. Interestingly, our results show that temperature can be as important as PSMs at ecologically relevant concentrations in limiting nutrient intake by herbivores in captivity. The effects of temperature are often overlooked in wildlife nutritional studies, so our results point to a key limitation of habitat assessments that do not link nutrient and PSM intake with current and future climate. In particular, our results indicate that future climate change, including warming average temperatures and heat wave events, effectively alter the nutritional landscape from the perspective of herbivores.

The effect of T_a on the intake of diets containing PSMs depended on the length of temperature exposure (Figures 3 and 4). In line with expectations, brushtail possums eat less when their food contains flavanone. Here we showed that this deterrent effect was also influenced by T_a. The deterrent effect of flavanone on brushtail possum intake was greater in possums exposed to higher temperatures for a week rather than a day. The difference in effects of T_a on intake by ringtail possums between overnight exposure and week-long exposure were more pronounced. Following overnight exposure to different T_a's, intake of high sideroxylonal leaves was not changed by increasing T_a. However, following a week of exposure to different temperatures, warm temperatures significantly reduced intake of high sideroxylonal leaves. The intake of basal diet and low sideroxylonal leaves by possums was also decreased as T_a increased, demonstrating that ambient temperature influences intake even when PSMs do not limit feeding. Below, we discuss the potential physiological mechanisms that underpin our results, and consider the importance of predicting the effects of rising T_a's on the feeding ecology of herbivorous mammals.

We formulated our predictions of feeding behaviour based on previous work indicating that hepatic detoxification rates of possums are reduced by week-long exposure to warm T_a's (Chapter 2). That work focussed on Alfaxalone, a hepatically metabolised anaesthetic, as a proxy for PSM metabolism. In other species, Alfaxalone is metabolised by cytochrome P450 enzymes in the liver (Warne et al. 2015), although there has been no analysis of metabolic pathways in possums. The consistency of the results between the change in the rate of Alfaxalone in possums with T_a and the feeding responses in the current study suggest that possums ate less of the diets containing PSMs at higher T_a because their rate of PSM metabolism was reduced. The effects of temperature on

tolerance of mammals to dietary PSMs, like those observed in this study, have also been reported by others as TDT (Kurnath et al. 2016).

At its simplest, the mechanism that underpins TDT is the ability of animals to dissipate heat. Both the digestion of food and the metabolism of PSMs produces metabolic heat (Beale et al. 2017). When T_a is high, especially when coupled with high humidity, dissipation of excess heat to the environment is difficult, even when using evaporative cooling (Cannon and Nedergaard 2011). As a consequence, physiological processes that generate heat may be down regulated in hot conditions to prevent hyperthermia (Speakman and Król 2010). This "heat dissipation limitation" is one possible explanation for the reduced capacity to detoxify xenobiotics, resulting in TDT, and hence a reduced voluntary intake of PSMs with an increase in T_a. In the future, it would be useful to titrate the exact amount of particular PSMs that animals are willing to eat at different temperatures to produce dose response curves.

The pattern of intake for brushtail and ringtail possums were almost identical in the long exposure experiment, but if contrasted with the short exposure experiment, then the magnitude of difference was greater in ringtail possums. This could be due to ringtails eating a leaf diet which would have included a mixture of other undetermined PSMs, or perhaps the larger body mass of brushtail compared to ringtail possums. Body mass is also cause for caution when comparing these results with previous studies of TDT, because most previous studies have been carried out on rodents. Unlike marsupials, rodents allow their body temperature to fluctuate when faced with a toxic challenge (Gordon et al. 2014). These changes in body temperature can affect hepatic metabolism of toxicants (Gordon et al. 2014). Gordon et al (2014) reviewed thermoregulatory responses in mice exposed to toxicants and found that mice housed at warmer temperatures were more susceptible to toxicity when they were unable to seek cooler microclimates. This suggests a similar problem to PSM ingestion (excessive metabolic heat production) but a different solution (regulated hypothermia). We did not measure the body temperature of possums during these feeding experiments, but it would be interesting to test whether there were metabolic changes in heat production in response to PSMs, or if the changes in intake were effective in countering hyperthermia. There was little opportunity for behavioural thermoregulation in the constant temperature rooms. In the wild, daytime temperatures can be buffered by

dens. Brushtail possums choose den sites that are on average 1.6˚C cooler than the T_a (Isaac et al. 2008). However, ringtail possums sometimes nest in dreys (a hollow mass of fine twigs and dry vegetation) rather than tree hollows and there has been no research on what thermal buffering these provide.

In addition to influencing the intake of diets containing high concentrations of PSMs, ambient temperature also affected intake of low-PSM diets in both possum species. In fact, possums maintained on low-PSM diets ate less food than required for weight maintenance at high T_a. Our respirometry data for ringtail possums showed a decrease in metabolic rate of resting, unfasted animals as temperature increased between 4˚C and 35˚C. An increase in temperature from 10˚C to 18˚C caused a far greater drop in RMR compared to the increase from 18˚C to 26˚C. However, food intake declined more dramatically when temperature was increased from 18˚C to 26˚C in the short exposure studies compared to the decline in intake at 18˚C compared to 10˚C, and in the long exposure studies the declines were equivocal. These effects reinforce the idea that even moderately high temperatures can cause limitations to food intake, such that animals eat up to their limit (imposed by HDL) rather than to their requirements. In domestic animals, this is regularly observed, with cattle (e.g.Bernabucci et al. 1999; Ominski et al. 2002), pigs (e.g.Close et al. 1971; Renaudeau et al. 2002), and poultry (e.g.Baziz et al. 1996; Dale and Fuller 1979), all known to reduce food intake at high T_a despite increased metabolic rates according to their TNZ.

The conventional TNZ, as proposed by Scholander (1955), is defined using changes in metabolic rate to indicate an UCT and LCT, between which the metabolic rate is low and constant. In many studies, an area of thermoneutrality is estimated by eye, however statistically adding a breakpoint in RMR at 19.3 °C was not significantly better than fitting a curve to our data. In addition, although body temperature increased at T_a's above 26.2 °C, there was no significant increase in metabolic rate, and statistically fitting a break point was no better than fitting a curve. If a possum's body temperature is increased, we question whether this animal is "thermoneutral". We therefore also question whether the UCT of the TNZ should be defined only by an increase in MR, or by whatever parameter first indicates that thermoregulatory stress has occurred. Furthermore, while we can detect where an UCT and LCT of the classical "broken stick" model may be, our data suggest that physiological adjustments in response to T_a occur

continuously. We agree with King (1964) that, while breakpoint regressions provide a useful way to statistically determine a traditional broken stick representation of thermoneutrality, in reality, this model is oversimplified and the transition zones are actually curvilinear.

In our measurements of thermal tolerance, we did not fast the animals, because ringtail possums are small (less than 1 kg), caecotrophic, and are prone to developing gastrointestinal disturbances when stressed (Vogelnest and Woods 2008). Since possums do not go for long periods of time without eating in the wild, our results are relevant indicators of temperature responses in wild animals. Furthermore, our lowest estimates of metabolic rates are lower than those proposed in previous experimental work that involved fasted animals (Munks 1990). Across longer time scales, animals are predicted to adapt to warming environments through changes in basal metabolism and thermal conductance (Fristoe et al. 2015). While we have said that the boundaries of the TNZ are most likely curvilinear, estimating when temperatures are sufficiently high to necessitate heat reduction mechanisms is still useful. A new approach to defining the UCT, could be to measure when voluntary intake of food is no longer determined by requirements, but instead by limitations to heat dissipation, in the absence of PSMs. In this way we can integrate how energy is being balanced in terms of both food and heat in a meaningful sense for future adaptation to changing temperatures. Animals eating less in the heat, must either do less, or must be more efficient in how they utilise what they do eat. Or it is possible in the field this may translate to animals seeking out cooler times to feed, cooler microclimates, or cooler regions if ingesting plants rich in PSMs (Bryant et al. 1994; Mole et al. 2016).

In this paper, we have shown the importance of considering temperature and food intake together, especially in the context of a diet rich in PSMs. This information could be used to better inform conservation efforts. For example, mechanistic niche models use the physiological parameters important to an animal, including estimates of thermal tolerance and diet selection, to predict future distributions under different climate scenarios (Kearney and Porter 2009). Until now, the importance of even seemingly mild changes in temperature for reducing food intake, and the interrelationship between temperature exposure and PSM intake, had not been fully recognised in wild herbivores. For instance, the closely related western ringtail possum (*Pseudocheirus*

peregrinus occidentalis) is now considered vulnerable, having experienced substantial population declines, and it appears to be particularly sensitive to warm temperatures (Jones et al. 1994; Yin 2006). As a result, conservation efforts have attempted to model the habitat requirements of western ringtail possums (Shedley and Williams 2014). But while these models consider both plant chemistry and T_a to be important, they are also considered static and distinct from each other (Shedley and Williams 2014). It is therefore likely that the report underestimates the effective loss of highly suitable habitat, the area of forest needed to sustain individuals, and the impact of warming temperatures. The same is true for other more phylogenetically distinct herbivore species, such as the giant panda (*Ailuropoda melanoleuca*). The giant panda is heat sensitive and consumes a bamboo diet rich in cyanogenic glycosides (GB et al. 1985; Huang et al. 2016). Mechanistic modelling has been used to determine whether the areas considered suitable habitat will be reduced due to changing thermal environments, or due to loss of food due to climate change (Yuke et al. 2018), when in fact one may dramatically accelerate the other by decreasing the tolerance of pandas to PSMs. While still valuable, these models could be further improved by considering the effects of temperature directly on herbivore nutrition.

We suggest that a decrease in PSM tolerance in response to warmer T_a's may be induced by multiple, yet to be elucidated mechanisms, but that our results are consistent with previous TDT studies in wild herbivores (e.g. Dearing et al. 2008; Kurnath et al. 2016). Both short temperature fluctuations and longer heat waves are predicted to increase in frequency and severity in the natural range of possums as the climate changes (CSIRO and BOM 2015). Our results indicate that shorter temperature fluctuations may be less harmful than week-long heat waves to herbivores consuming toxic plants. However, increased T_a's across longer timescales may reduce nutrient intake in possums, with flow on effects to populations. Alternatively, possums may be forced to feed at cooler times, seek out cooler microclimates, or be more selective for individual trees with lower PSM concentrations (if present) within their environment, leading to a change in plant-herbivore interactions within ecosystems, and a need for conserved areas to encompass this variety in plant chemistry. At broader scales this may mean herbivores living in cooler environments, such as snowshoe hares are able to

maintain diets higher in PSMs. The importance of PSMs in determining herbivore food intake cannot be separated from the direct effects of T_a on herbivore physiology.

This work was approved by the Australian National University Animal Experimentation Ethics Committee and conforms to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

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References

Baziz HA, Geraert PA, Padilha JCF, Guillaumin S (1996) Chronic heat exposure enhances fat deposition and modifies muscle and fat partition in broiler carcasses. Poult. Sci 75:505-513

Beale PK, Marsh KJ, Foley WJ, Moore BD (2017) A hot lunch for herbivores: physiological effects of elevated temperatures on mammalian feeding ecology. Biol Rev Camb Philos Soc. doi: 10.1111/brv.12364

Bernabucci U, Bani P, Ronchi B, Lacetera N, Nardone A (1999) Influence of short- and long-term exposure to a hot environment on rumen passage rate and diet digestibility by Friesian heifers. J Dairy Sci 82:967-973

Boyle R, Dearing MD (2003) Ingestion of juniper foliage reduces metabolic rates in woodrat (Neotoma) herbivores. Zoology 106:151-158. doi: https://doi.org/10.1078/0944-2006-00109

Boyle RR, McLean S (2004) Constraint of feeding by chronic ingestion of 1,8-cineole in the brushtail possum (*Trichosurus vulpecula*). J Chem Ecol 30:757-775

Bryant J, Swihart R, Reichardt P, Newton L (1994) Biogeography of woody plant chemical defense against snowshoe hare browsing: comparison of Alaska and Eastern North America. Oikos 70. doi: 10.2307/3545776

Camp MJ, Shipley LA, Milling CR, Rachlow JL, Forbey JS (2018) Interacting effects of ambient temperature and food quality on the foraging ecology of small mammalian herbivores. J Therm Biol 71:83-90. doi: https://doi.org/10.1016/j.jtherbio.2017.10.021

Cannon B, Nedergaard J (2011) Nonshivering thermogenesis and its adequate measurement in metabolic studies. J Exp Biol 214:242-253

Close WH, Mount LE, Start IB (1971) The influence of environmental temperature and plane of nutrition on heat losses from groups of growing pigs. Anim Prod 13:285-294

Connors PK, Malenke JR, Dearing MD (2017) Ambient temperature-mediated changes in hepatic gene expression of a mammalian herbivore (*Neotoma lepida*). Mol Ecol 26:4322-4338. doi: 10.1111/mec.14192

CSIRO, BOM (2015) Climate change in Australia technical report. In: Whetton P (ed) Climate change in Australia information for Australia's natural resource management regions: technical report. CSIRO and Bureau of Meteorology, Australia

Dale NM, Fuller HL (1979) Effect of low temperature, diet density, and pelleting on the preference of broilers for high fat rations. Poult. Sci 58:1337-1339

Dawson TJ, Olson JM (1988) Thermogenic capabilities of the opossum *Monodelphis domestica* when warm and cold acclimated - Similarities between American and Australian marsupials. Comp. Biochem. Physiol. A 89:85-91. doi: 10.1016/0300-9629(88)91143-7

Dearing MD (2013) Temperature-dependent toxicity in mammals with implications for herbivores: a review. J Comp Physiol B 183:43-50

Dearing MD, Foley WJ, McLean S (2005) The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. Annu Rev Ecol Evol Syst 36:169-189

Dearing MD, Forbey JS, McLister JD, Santos L (2008) Ambient temperature influences diet selection and physiology of an herbivorous mammal, *Neotoma albigula*. Physiol Biochem Zool 81:891-897

Foley WJ, Moore BD (2005) Plant secondary metabolites and vertebrate herbivores – from physiological regulation to ecosystem function. Curr Opin Plant Biol 8:430-435. doi: 10.1016/j.pbi.2005.05.009

Forbey JS, Dearing MD, Gross EM, Orians CM, Sotka EE, Foley WJ (2013) A pharm-ecological perspective of terrestrial and aquatic plant-herbivore interactions. J Chem Ecol 39:465-480. doi: 10.1007/s10886-013-0267-2

Forbey JS, Liu R, Caughlin TT, Matocq MD, Vucetich JA, Kohl KD, Dearing MD, Felton AM (2018) Review: Using physiologically based models to predict population responses to phytochemicals by wild vertebrate herbivores. Animal 12:s383-s398. doi: 10.1017/s1751731118002264

Fristoe TS, Burger JR, Balk MA, Khaliq I, Hof C, Brown JH (2015) Metabolic heat production and thermal conductance are mass-independent adaptations to thermal environment in birds and mammals. Proc. Natl. Acad. Sci. U. S. A 112:15934-15939. doi: 10.1073/pnas.1521662112

GB S, Hu J, Pan W, Zhu J (1985) The Giant Pandas of Wolong. . The Quarterly Review of Biology 60:524-525. doi: 10.1086/414647

Gordon CJ, Johnstone AFM, Aydin C (2014) Thermal stress and toxicity. Compr Physiol 4:995–1016

Gordon CJ, Mohler FS, Watkinson WP, Rezvani AH (1988) Temperature regulation in laboratory mammals following acute toxic insult. Toxicology 53:161-178

Herd RM, Oddy VH, Richardson EC (2004) Biological basis for variation in residual feed intake in beef cattle. 1. Review of potential mechanisms. Australian Journal of Experimental Agriculture 44:423-430. doi: 10.1071/EA02220

Huang H, Yie S, Liu Y, Wang C, Cai Z, Zhang W, Lan J, Huang X, Luo L, Cai K, Hou R, Zhang Z (2016) Dietary resources shape the adaptive changes of cyanide detoxification function in giant panda (*Ailuropoda melanoleuca*). Scientific reports 6:34700-34700. doi: 10.1038/srep34700

Intergovernmental Panel on Climate Change (2007) Projections of Future Changes in Climate. Accessed: 15 Feb 2018

Isaac J, De Gabriel J, Goodman B (2008) Microclimate of daytime den sites in a tropical possum: implications for the conservation of tropical arboreal marsupials. Anim Conserv 11:281-287. doi: 10.1111/j.1469-1795.2008.00177.x

Jones BA, How RA, Kitchener DJ (1994) A field study of *Pseudocheirus occidentalis* (Marsupialia : Petauridae) II. Population studies. Wildl Res 21:189-201

Kaplanski J, Ben-Zvi Z (1980) Effect of chronic heat exposure on *in-vitro* drug metabolism in the rat. Life Sci 26:639–642

Kearney M, Porter W (2009) Mechanistic niche modelling: combining physiological and spatial data to predict species' ranges. Ecol. Lett. 12:334-350. doi: 10.1111/j.1461-0248.2008.01277.x

Kearney MR, Simpson SJ, Raubenheimer D, Kooijman SALM (2012) Balancing heat, water and nutrients under environmental change: A thermodynamic niche framework. Funct Ecol 27:950-966

Kearney MR, Wintle BA, Porter WP (2010) Correlative and mechanistic models of species distribution provide congruent forecasts under climate change. Conservation Letters 3:203-213. doi: 10.1111/j.1755-263X.2010.00097.x

Keplinger ML, Lanier GE, Deichmann WB (1959) Effects of environmental temperature on the acute toxicity of a number of compounds in rats. Toxicol Appl Pharmacol 1:156-161

King JR (1964) Oxygen consumption and body temperature in relation to ambient temperature in the White-crowned Sparrow. Comparative Biochemistry and Physiology 12:13-24

Kurnath P, Dearing MD (2013) Warmer ambient temperatures depress liver function in a mammalian herbivore. Biol Lett 9:20130562. doi: 10.1098/rsbl.2013.0562

Kurnath P, Merz ND, Dearing MD (2016) Ambient temperature influences tolerance to plant secondary compounds in a mammalian herbivore. Proc R Soc Lond B 283:20152387

Lawler IR, Foley WJ, Eschler BM (2000) Foliar concentration of a single toxin creates habitat patchiness for a marsupial folivore. Ecology 81:1327-1338. doi: 10.1890/0012-9658(2000)081[1327:FCOAST]2.0.CO;2

Marsh KJ, Saraf I, Hocart CH, Youngentob K, Singh I-P, Foley WJ (2019) Occurrence and distribution of unsubstituted B-ring flavanones in Eucalyptus foliage. Phytochemistry 160:31-39. doi: 10.1016/j.phytochem.2019.01.005

Marsh KJ, Wallis IR, Andrew RL, Foley WJ (2006a) The detoxification limitation hypothesis: where did it come from and where is it going? J Chem Ecol 32:1247-1266. doi: 10.1007/s10886-006-9082-3

Marsh KJ, Wallis IR, Foley WJ (2005) Detoxification rates constrain feeding in common brushtail possums (*Trichosurus vulpecula*). Ecology 86:2946-2954. doi: 10.1890/05-0303

Marsh KJ, Wallis IR, McLean S, Sorensen JS, Foley WJ (2006b) Conflicting demands on detoxification pathways influence how common brushtail possums choose their diets. Ecology 87:2103-2112

Marsh KJ, Yin B, Singh IP, Saraf I, Choudhary A, Au J, Tucker DJ, Foley WJ (2015) From leaf metabolome to in vivo testing: identifying antifeedant compounds for ecological studies of marsupial diets. J. Chem. Ecol. 41:513-519. doi: 10.1007/s10886-015-0589-3

Mole MA, Rodrigues DÁraujo S, van Aarde RJ, Mitchell D, Fuller A (2016) Coping with heat: behavioural and physiological responses of savanna elephants in their natural habitat. Conservation Physiology 4. doi: 10.1093/conphys/cow044

Muggeo VMR (2003) Estimating regression models with unknown break‐points. Stat Med 22:3055-3071. doi: doi:10.1002/sim.1545

Munks SA (1990) Ecological energetics and reproduction in the common ringtail possum, *Pseudocheirus peregrinus* (Marsupialia: Phalangeroidea). PhD, University of Tasmania, Hobart

Oliver AJ, King DR (1983) The influence of ambient temperatures on the susceptibility of mice, guinea pigs and possums to compound 1080. Aust. Wildl. Res. 10:297-301

Ominski KH, Kennedy AD, Wittenberg KM, Nia SAM (2002) Physiological and production responses to feeding schedule in lactating dairy cows exposed to short-term, moderate heat stress. J Dairy Sci 85:730-737

Renaudeau D, Collin A, Yahav S, de Basilio V, Gourdine JL, Collier RJ (2012) Adaptation to hot climate and strategies to alleviate heat stress in livestock production. Animal 6:707-728. doi: 10.1017/S1751731111002448

Renaudeau D, Quiniou N, Dubois S (2002) Effects of high ambient temperature and dietary protein level on feeding behavior of multiparous lactating sows. Anim Res 51:227-243. doi: 10.1051/animres:2002020

Saraf I, Marsh KJ, Vir S, Foley WJ, Singh IP (2017) Quantitative analysis of various B-ring unsubstituted and substituted flavonoids in ten Australian species of *Eucalyptus*. Natural product communications 12:1695-1699

Schäfer K (1998) Accelerated solvent extraction of lipids for determining the fatty acid composition of biological material. Anal Chim Acta 358:69-77. doi: 10.1016/S0003-2670(97)00587-4

Scholander PF (1955) Evolution of climatic adaptation in homeotherms. Evolution 9:15-26

Settivari RS, Evans TJ, Eichen PA, Rottinghaus GE, Spiers DE (2008) Short- and long-term responses to fescue toxicosis at different ambient temperatures. J Therm Biol 33:213-222

Shedley E, Williams K (2014) An assessment of habitat for western ringtail possum (*Pseudocheirus occidentalis*). Department of Parks and Wildlife

Speakman JR, Król E (2010) Maximal heat dissipation capacity and hyperthermia risk: neglected key factors in the ecology of endotherms. J Anim Ecol 79:726-746

Torregrossa A-M, Dearing MD (2009) Nutritional toxicology of mammals: regulated intake of plant secondary compounds. Funct Ecol 23:48-56. doi: 10.1111/j.1365-2435.2008.01523.x

Vogel KP (1999) Evaluation of a filter bag system for NDF, ADF, and IVDMD forage analysis. Crop Sci 39:276-279. doi: 10.2135/cropsci1999.0011183X003900010042x

Vogelnest L, Woods R (2008) Medicine of Australian Mammals, 1 edn. CSIRO Publishing, Canberra

Warne LN, Beths T, Whittem T, Carter JE, Bauquier SH (2015) A review of the pharmacology and clinical application of alfaxalone in cats. T Vet J 203:141-148. doi: 10.1016/j.tvjl.2014.12.011

Wiggins NL, Marsh KJ, Wallis IR, Foley WJ, McArthur C (2006) Sideroxylonal in *Eucalyptus* foliage influences foraging behaviour of an arboreal folivore. Oecologia 147:272-279

Yin H (2006) The metabolic and hygric physiology of Western Ringtail Possum (*Pseudocheirus occidentalis*). Honours thesis, Curtin University of Technology, Perth

Youngentob KN, Wallis IR, Lindenmayer DB, Wood JT, Pope ML, Foley WJ (2011) Foliage chemistry influences tree choice and landscape use of a gliding marsupial folivore. J Chem Ecol 37:71-84

Yuke Z, D. MP, Qiongyue Z, P. PW, Jianghong R (2018) An ecophysiological perspective on likely giant panda habitat responses to climate change. Global Change Biol 24:1804-1816. doi: doi:10.1111/gcb.14022

Chapter 4

Macronutrient selection changes with ambient temperature in a mammalian herbivore

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Abstract

Average global temperatures and the number of days with extreme heat are increasing and there are many effects of temperature on feeding in mammals. We tested whether housing in each of three ambient temperatures spanning the thermoneutral zone (10˚C, 18˚C, and 26˚C) influenced the voluntary intake of protein by a marsupial herbivore, the common brushtail possum (*Trichosurus vulpecula*). At each temperature, possums were offered a continuous choice of two diets containing different amounts of protein (57% vs 8%) for one week. Animals chose to eat a diet lower in protein to non-protein (P:NP, 0.20) when held at 26˚C compared to that at both 10˚C and 18˚C (0.22). Since detoxification of plant secondary metabolites imposes a protein cost on animals, we then studied whether addition of the monoterpene 1,8-cineole to the food changed the effect of ambient temperature (10˚C and 26˚C) on food choice. Cineole reduced overall food intake by possums but they no longer chose a different P:NP ratio when exposed to different temperatures and instead always opted for a diet with higher P:NP (0.19 for cineole diet vs 0.15 for basal diet). These experiments show that animals can choose non-randomly between diets of different macronutrient compositions and the proportion of P:NP chosen is influenced by ambient temperature and by secondary plant metabolites. Protein is critical for reproductive success in these animals and high ambient temperatures at critical times in the breeding cycle may limit the viability of some animal populations in the future.

Introduction

Ambient temperature is a known determinant of the feeding behaviour and physiology of endothermic herbivores (Beale et al. 2017). The most conspicuous effect is an increase in food intake accompanying an increase in metabolic rate at cool ambient temperatures (Scholander 1955). However ambient temperature can also influence the digestion, metabolism and excretion of nutrients and plant secondary metabolites ((PSMs)Baumgard and Rhoads 2012; Baumgard et al. 2011; Hammond and Diamond 1994).

The link between nutrition and elevated ambient temperature has been best investigated in domestic species (e.g. Ferguson and Gous 1997) particularly in intensive production systems such as dairy and poultry. Observations have shown that animals defer feeding during the hot part of the day and eat less overall in hot conditions. This comes despite an increase in metabolic rate due to thermoregulatory demands above the thermoneutral zone and is often accompanied by reduced weight and productivity. Heat stress can also negatively affect energy balance and nutrient utilisation including reduced lipolysis resulting in fat deposition (Rhoads et al. 2013). Several dietary manipulations have been proposed to ameliorate these effects including supplementation with lipoic acid to mimic insulin or with chromium to enhance its effects, electrolyte supplementation to counter losses in sweat, and alterations to the macronutrient composition of the diet (Renaudeau et al. 2012; West 1999). The primary motivation for altering macronutrient composition in heat-stressed animals is to minimize heat from digestion and metabolism of food, which contributes to the internal heat load but also acts as a satiety signal. This diet-induced thermogenesis (DIT) is dependent on meal size, with larger meals generating more heat, but also on the macronutrient composition of the diet since the different macronutrients produce different amounts of heat during digestion and metabolism (Beale et al. 2017). Diet-induced thermogenesis (DIT) is greatest for protein and least for fats, with soluble carbohydrate falling between. DIT from fermentation is variable and depends on particulars of digestive anatomy and physiology, with heat arising from both heat of fermentation and from the less efficient utilisation of short-chain fatty acids compared to glucose (Renaudeau et al. 2012; Secor 2009; Westerterp 2004). In cold conditions,

DIT from carbohydrate and fat-rich diets can be considered to be compensatory thermogenesis; i.e. it reduces the demand for specific thermogenesis. High-protein diets are still tolerated and can provide production advantages. To maximise production under conditions that impose heat stress, diets are formulated for production animals that minimize thermogenic fiber and protein, while maintaining maximal energy intake (West 1999). These formulated diets used in intensive production systems do not allow animals to use diet choice to balance nutrients as wild herbivores would be expected to do, but instead aim to replicate the way an herbivore would balance nutrients at different ambient temperatures to maximize voluntary intake.

Unlike domestic animals, wild herbivores choose from diverse foods in a complex nutritional landscape to meet their nutrient and thermoregulatory requirements. As wild animals are also aiming to maximize production in the form of growth and reproduction, we might expect similar changes in diet composition with temperature would be helpful to wild herbivores facing heat challenge. However, detecting temperature-induced changes to nutrient selection by wild herbivores is complicated by changes in plant composition that may occur seasonally or with temperature fluctuations and by the presence of plant secondary metabolites (PSMs) which may deter feeding and must be detoxified prior to excretion. Plant secondary metabolites can be as important as nutrients in driving the feeding decisions of herbivores (Dearing et al. ; Foley et al. 1999; Forbey and Foley 2009), and metabolism of PSMs by herbivores can also be influenced by ambient temperature (Beale et al. 2017). Temperature-dependent toxicity is a phenomenon whereby many xenobiotics, including some PSMs, are metabolised more slowly by endothermic vertebrates when they are exposed to them at warmer ambient temperatures, resulting in reduced intake of PSMs and hence of food containing PSMs (Beale unpublished data, Dearing 2013; Kurnath and Dearing 2013; Kurnath et al. 2016).

Macronutrient and PSM intake are often unavoidably linked and should not be viewed in isolation (Villalba and Provenza 2005). For example, PSMs can reduce the availability of nutrients (e.g. tannins binding to protein), and nutrients can either ameliorate the effects of PSMs or enhance an herbivores' capacity to metabolize PSMs (Iason 2005). For example, lambs infused intraruminally with different PSMs showed varied responses in terms of overall food intake and macronutrient preference depending on the PSM

(Villalba et al. 2002c). In sheep and goats, diets supplemented with protein increased voluntary intake of tannins and supplementary protein also positively influenced intake of shrubs such as sagebrush (Villalba et al. 2002a; Villalba et al. 2002b). Increasing dietary protein concentration also allowed increased intake of cineole and benzoate by brushtail possums, in the later instance by offsetting a protein cost of detoxification (Au et al. 2013). Therefore, reduced intake of protein with an increase in ambient temperature could also reduce voluntary intake of PSMs in addition to TDT.

Here we investigate how a generalist marsupial folivore, the common brushtail possum *(Trichosurus vulpecula*), alters the macronutrient composition of its diet when exposed to different ambient temperatures, and also to the PSM cineole, which it encounters in its natural diet. We offered possums a choice between two isocaloric diets differing in protein and carbohydrate composition. We predicted that possums would not only eat less, but that they would assemble a diet with a lower protein concentration under warm conditions than under cool conditions. However, Previous authors found that if offered diets higher in protein, brushtail possums were also able to tolerate more PSMs (Au et al. 2013). Although, some studies using other small mammalian herbivores have not found an effect of temperature on cineole intake (Camp et al. 2018), we anticipated that the addition of cineole would reduce intake of food at all temperatures (Boyle and McLean 2004), we tested whether the need to manage DIT would over-ride the need to consume more protein for detoxification at different temperatures. We predicted that when cineole was included in the diet, possums would ingest less but of a higher protein diet.

Understanding how temperature drives changes in the selection of macronutrients will contribute to better predictions of how herbivore populations will respond to hotter climates. More frequent and more severe heat waves are predicted across the entire range of brushtail possums, in addition to warmer average temperatures. Further, while there is some variation in results, the nutritional quality of food trees is predicted to decline. Therefore, understanding how herbivores adjust their nutritional decision making with changes to ambient temperature is imperative for conservation efforts.

Methods

Animals, housing and diets

Twelve adult male common brushtail possums (*Trichosurus vulpecula*) were trapped in cage traps baited with apple on the Australian National University campus (Canberra, 35.2809° S, 149.1300° E) in November 2016. The age (by tooth wear, Pahl 1987; Winter 1980) and condition of all possums was checked prior to the feeding experiments, to insure that only healthy adults were included. Possums were housed in cages (90 x 70 x 152cm) with an open three-sided nest platform (18 x 18 x 34cm). This was to prevent temperature buffering by the nest box. The cages were placed inside constant temperature rooms (± 2°C) with a 12:12 h light cycle. Over a period of five weeks, possums were transitioned onto a compounded diet of fruit and cereals (see below). This was prepared fresh daily and fresh water was always available *ad libitum*. Food was offered at 1630 and collected at 0900 daily. Possums were weighed weekly.

Throughout both experiments possums were offered a choice between two isocaloric diets. Both diets contained 39% pureed apple, 29% pureed banana, 9% pureed carrot, 3% purified wood celluose ("Just Fiber", International Fiber Corporation North Tonawanda, NY), 10% ground rice hulls, 2% rolled oats, 2% ground Lucerne hay, 1.47% vegetable oil, 0.25% salt, 0.25% dicalcium phosphate, and 0.03% vitamin and mineral supplement, all on a wet matter basis. Casein and sugar were substituted for each other to generate a higher protein diet (3.99% acid casein and 0.01% sugar) and a lower protein diet (0.01% casein and 3.99% sugar). The high-protein diet was coloured red with a food dye (Queen brand) and always offered on the right-hand side of the aviary. The low-protein diet was coloured blue (Queen brand) and always offered on the left-hand side. The colouring allowed dropped food to be weighed back as the correct diet, and preliminary testing showed that the colour did not influence food choice (data not shown). The diets were consistently offered on the same side, as our primary goal was to test the influence of temperature on macronutrient composition rather than the ability of the possums to find the diets. A side bias could be eliminated based on whether temperature treatments altered diet selection.

Chemical analysis of diets

Ingredients used to formulate the diets have natural variation in their nutritional composition leading to slight differences in the composition of diets, even when intended to be identical, so the actual composition of the diets was measured for both experiments. The total nitrogen (N), and hence protein (N x 6.25), concentration of diets was determined using a LECO TruSpec® CN analyser. Accelerated solvent extraction (Thermo Scientific, Sunnyvale CA) in a Dionex
ASE350 accelerated solvent extraction machine was used to determine the fat content of diets. 0.5 g of sample was mixed with 2 g of preparative diatomaceous earth and extracted in a 10 ml stainless steel cell in petroleum ether (40-60 degress bp). The standard extraction method was used, with samples heated to 120°C for 6 minutes, followed by a 2 minute static hold. Cells were flushed with 60% of cell volume and purged with nitrogen for 60 sec before a second cycle. The extracted solution was collected into pre-weighed bottles, which were dried under nitrogen and then dried in a 102 °C oven for 2 hours prior to weighing. Neutral detergent fibre (NDF) was determined using the an ANKOM fibre bag protocol (ANKOM Technology, Method 6). The total carbohydrate content of diets was analysed using the anthrone method. However, this analysis needs to be repeated so final data are not available at this time. Therefore, the carbohydrate content presented here are those calculated by subtraction. They are very similar to carbohydrate content estimated from the soluble fraction of the available nitrogen assay bringing confidence in their accuracy. Once the final carbohydrate values are determined from the anthrone assay, these will be used to reanalyse the data for publication.

Table 1: Nutritional composition of the diets offered in each experiment.

	EXPERIMENT 1		EXPERIMENT 2	
	High Protein: Non-protein	Low Protein: Non-protein	High Protein: Non-protein	Low Protein: Non-protein
Neutral detergent fiber %	51.65	42.47	49.09	43.18

Fat %	1.81	1.73	1.23	1.97
Protein %	17.60	4.53	18.09	4.50
Carbohydrate %	28.94	51.27	31.59	50.35

Macronutrient choice at different ambient temperatures

Possums were randomly allocated into three groups of four, and each group was kept at a constant temperature of either 10, 18 or 26 ± 2°C for seven days. These temperatures were chosen according to our previous respirometry measurements in ringtail possums indicating 10°C is below the thermoneutral zone, 18°C is around the lower critical temperature, and 26°C is around the upper critical temperature of the thermoneutral zone (Cooper, personal communication, Dawson 1969, Beale unpublished data). Dry matter intake (DMI) of the two diets was measured between 1630 and 0900 h daily by drying a subsample of the food offered at 80 °C (to determine the DM, 35% DM on average), as well as all food refusals. Thus, total daily DMI and diet composition (g protein and non-protein ingested; P:NP) was recorded across 7 days for each possum for each temperature. We have used "non-protein" to refer to the sum by weight of fats and soluble carbohydrates. The possums were then rotated between the temperature treatments for two more rounds of the experiment, using a randomized crossover design.

The effects of ambient temperature and cineole on macronutrient choice

All possums were housed at 18°C for one week prior to the experiment. Possums were then randomly allocated between two groups. One group was kept at 26 ± 2°C for seven nights, while the other was kept at 10 ± 2°C. Possums were offered the two diets differing in macronutrient composition without addition of any PSM for the first six nights. DMI and diet preferences were measured on the fifth and sixth nights and the mean was taken as an estimate of intake of the basal diets. On the seventh night 1,8-

cineole was added to both diets at a concentration of 2.45% WM (~7% DM). DMI and diet preferences were measured again.

Statistical analyses

Results were analyzed in R (Packages: lme4, lmerTest, emmeans, pbkrtest, emmeans) and plotted (packages: ggplot2, wesanderson, gridExtra, plyr). For both experiments, the ratio of protein to non-protein (P:NP) eaten was analysed using a generalized linear mixed model. In the first experiment, ratio of P:NP eaten was entered as the dependent variable and temperature was the independent variable. In the second experiment temperature and PSM and their interaction were included as the independent variables. Possum identity and experimental round were included as random variables with random intercepts for both models. The models were specified as having a gamma distribution with a log-link. An "equal intake scenario" was included in the model as an offset variable to determine if the possums chose randomly or non-randomly between the diets. This ratio of P:NP was calculated as if each possum ate the same amount of food as they did in the experiment, but chose equal amounts of each diet (Felton et al. 2016). The model was compared to a model containing only the random effects using an ANOVA. Each pairwise comparison, and P-values were gained using the package "emmeans".

The geometric framework of nutrition, developed by Simpson and Raubenheimer (Simpson and Raubenheimer 2001), was used to visualize the results. In this method, each axis represents intake of a selected nutrient, starting at an intake of zero at the origin. A nutritional rail is a line drawn out from the origin, for which any point along the line would have the same composition in terms of the nutrients represented by each axis. A larger meal would appear farther from the origin and a smaller meal would appear closer to the origin. In this experiment, the mean intake across replicates was plotted as a point to visualize different treatments. As animals were selecting from two diets, their intake could only fall on, or between, the nutritional rail of the two diets. If animals in different treatment groups selected different macronutrient composition, the points would appear on different nutritional rails. If they selected the same composition, but ate different amounts the points would appear on the same nutritional rail.

Results

Macronutrient choice and food intake at different ambient temperatures

Ambient temperature significantly affected intake of P:NP (χ^2 (2)= 20.57, p<0.001). Each pairwise comparison of P:NP intake at each temperature is reported in Table 2.

*Table 2: Model estimates and each pairwise comparison for temperatures and equal intake scenario in experiment 1. NS = Not significant, *** = P<0.001. Of the random effects possum identity accounted for 0.007 (sd = 0.08) and experimental round accounted for 0.0009 (sd = 0.03) of variance in the intake ratio, with a residual variance of 0.020 (sd = 0.14). Estimates are rounded to two decimal places.*

Comparison	Ratio Estimate	SE	10˚C	18˚C	26˚C
10˚C	0.22	0.02			
18˚C	0.22	0.02	NS		
26˚C	0.20	0.01	***	***	
Equal intake	0.26	0.003	***	***	***

Temperature also significantly affected DMI ($\chi^2(2) = 54.21$, $p<0.001$). Each pairwise comparison is shown in table 3. reducing intake from 87.14g at 10˚C to 83.94g at 18˚C and to 75.54g at 26˚C (P<0.05 in 10˚C-18˚C comparison, and P<0.001 in each 26˚C comparison).

*Table 3: Model estimates and each pairwise comparison for DMI at each temperature in experiment 1. NS = Not significant, *** = P<0.001. Of the random effects possum identity accounted for 155.66 (sd = 12.47) and experimental round accounted for 17.60 (sd= 4.195) of variance in the intake ratio, with a residual variance of 80.78 (sd = 8.98) Estimates are rounded to two decimal places.*

Comparison	Dry matter intake (g)	SE	10˚C	18˚C	26˚C
10˚C	87.13	4.75			
18˚C	83.90	4.75	NS (P=0.08)		
26˚C	75.52	4.75	***	***	

Although there was a trend for possums to eat less at 18 °C relative to 10 °C (Table 3; Figure 1), the composition of the diet that they selected at these temperatures was the same (Table2; Figure 1). Possums chose a diet lower in P:NP at 26°C compared to either of the other two temperatures (Table 2; Figure 1). At 26˚C possums also ate significantly less than at 10˚C and 18˚C (Table 3).

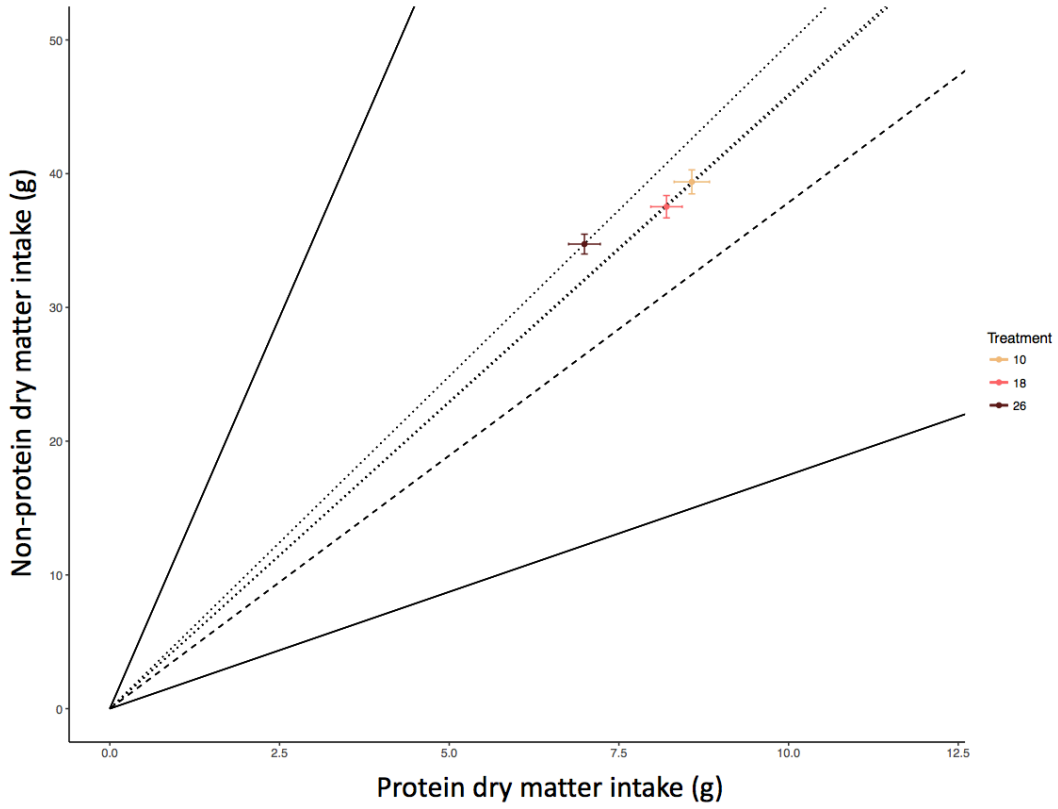

Figure 1: Mean +/-SE total protein vs non-protein (fats and carbohydrate) intake by brushtail possums (N = 12) exposed to three ambient temperatures (10˚C, 18˚C, and 26°C) for one week. The nutritional rails observed in the experiment are indicated by dotted lines, the dashed line indicates the expected nutritional rail if possums chose equally between the high and low protein diets. The two solid lines indicate the nutritional rail of each diet in isolation.

Macronutrient choice and food intake at different ambient temperatures with cineole

Cineole and temperature significantly influenced macronutrient selection by possums ($\chi2(3) = 43.13$, $p<0.001$). Each pairwise comparison is reported in Table 4.

*Table 4: Model estimate and each pairwise comparison of P:NP intake for temperatures and diets with and without cineole in experiment 2. NS = Not significant; P-value given, *** = P<0.001. Of the random effects possum identity accounted for 0.01(sd= 0.10) and experimental round accounted for 0.0009 (sd = 0.03) of variance in the intake ratio, with a residual variance of 0.02 (sd = 0.13). Ratio estimates are rounded to two decimal places.*

	Temp(˚C)	Protein: non-protein	SE	Basal		Cineole	
				10˚C	26˚C	10˚C	26˚C
Basal	10	0.15	0.01		NS(0.14)	***	***
	26	0.14	0.01			***	***
Cineole	10	0.19	0.02	***	***		NS(0.99)
	26	0.18	0.02	***	***		

The diet selected in the presence of cineole was higher in protein relative to carbohydrates and fat (P:NP) than the diets selected in the absence of cineole (Table 4; Figure 2). Diet composition did not differ significantly between 10˚C and 26**˚C** in either the presence or absence of cineole (Table 4; Figure 2). It is likely our experiment did not have enough power to detect the difference in P:NP with temperature visible in Figure 2 for the basal diet treatments as this was relatively small compared to the influence of cineole. When the basal diet was modelled in isolation, temperature had a significant effect as in experiment 1 (χ^2 (1)= 7.33, p=0.006). When the cineole diet was modelled in isolation the effect of temperature was non-significant (χ2 (1)= 0.13, p=0.91).

*Table 5: Model estimate and each pairwise comparison of DMI for temperatures and diets with and without cineole in experiment 2. NS = Not significant; P-value given, *** = P<0.001. Of the random effects possum identity accounted for 95.54 (sd = 9.774) and experimental round accounted for 3.92 (sd = 1.980) of variance in the DMI, with a residual variance of 84.10 (sd = 9.171). Estimates are rounded to two decimal places.*

	Temp(˚C)	Estimate of dry matter intake	SE	Basal		Cineole	
				10˚C	26˚C	10˚C	26˚C
Basal	10	92.14	3.88		NS(0.16)	***	***
	26	86.54	3.88			***	***
Cineole	10	54.37	4.48	***	***		NS(0.37)
	26	47.66	4.48	***	***		

Possums ate less when cineole was included in the diet (Table 5; Figure 2). Again it is likely our overall model was not powerful enough to detect the relatively small effect of temperature on intake. However when analysed in isolation increasing temperatures from 10˚C to 26˚C significantly reduced intake in both the basal treatment ($\chi 2$ (1)= 3.86, p=0.05) and the cineole treatment ($\chi 2$ (1)= 5.18, p=0.02).

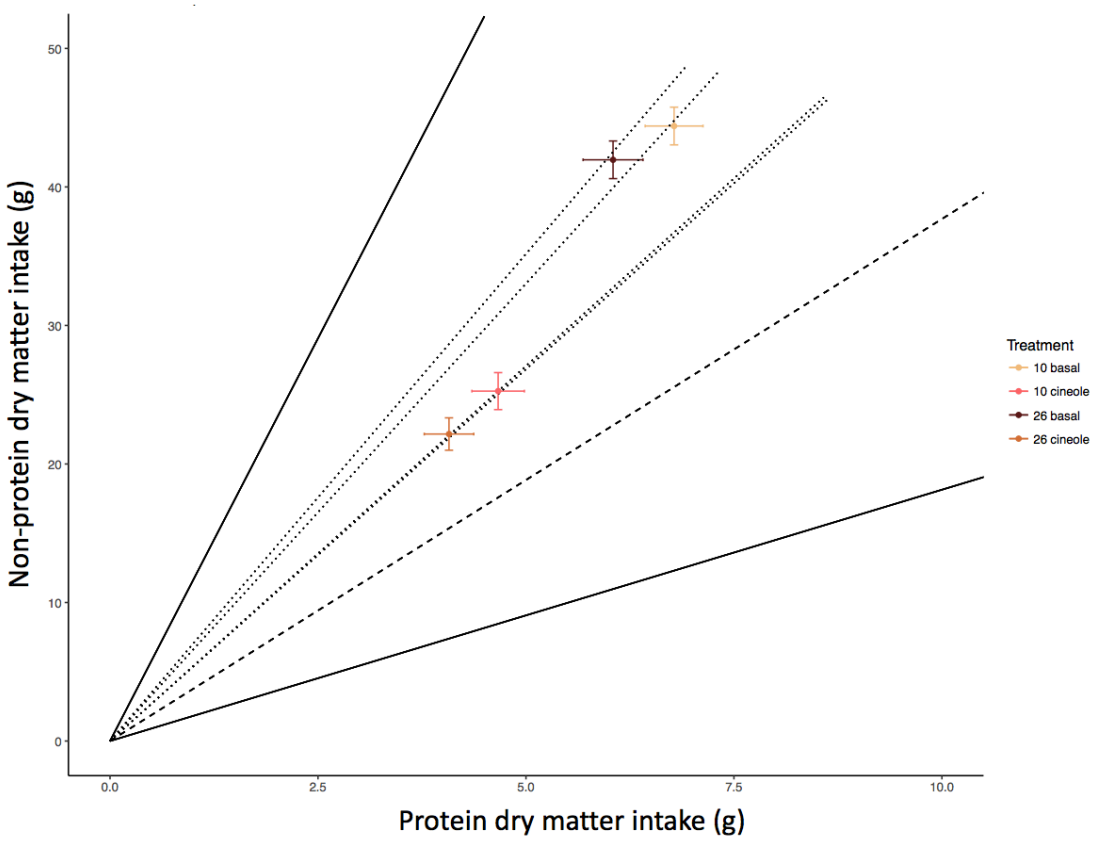

Figure 2: *Mean +/-SE protein vs non-protein (fats and carbohydrate) intake by brushtail possums housed at two temperatures (10 and 26°C) for a week and offered diets with or without cineole. The nutritional rails are indicated by dotted lines, the dashed line indicates the expected nutritional rail if possums chose randomly between the high and low protein diets. The two solid lines indicate the nutritional rail of each diet in isolation.*

Discussion

The major finding in this study was that common brushtail possums modified their selection of macronutrients at higher temperatures but that this effect was abolished when a plant secondary metabolite was included in the diet. This is an important finding because most previous studies have only focussed on the effect of global climate change on the nutritional composition of plants. Available protein is the critical determinant of reproductive success in brushtail possums (DeGabriel et al. 2009; Windley et al. 2013) and other herbivorous mammals (Allen and Ullrey 2004; McArt et al. 2009) and the finding that even modest changes in temperature lead to declines in

the voluntary intake of protein demonstrate that a warming climate could result in population declines.

The temperatures used in this experiment were based on respirometry data characterising the thermal tolerance of possums (Cooper, personal communication, Beale unpublished data, Dawson 1969`). Possums have greater energetic requirements when exposed to 10˚C compared to 18˚C. At 26˚C possum should have similar energetic requirements to 18˚C but are facing metabolic limitations due to an inability to dissipate excess heat. Our experiment demonstrated that possums respond to the limitations imposed by warmer ambient temperatures by adjusting their macronutrient selection. At 26˚C possums consumed less P:NP and less food (and hence energy) overall than at cooler temperatures. This is consistent with our hypothesis that due to HDL herbivores should aim to minimise DIT by reducing P:NP, and pertains to the digestion and hepatic metabolism of protein generating more DIT than the other macronutrients. In experiment 1 possums both absolutely and proportionately reduced protein intake in warm conditions. This is in direct contrast to how possums responded to cooler temperatures. At 10˚C compared to 18˚C possums compensated for increased energetic costs only by increasing their total food intake without changing the nutrient balance. This finding reinforces the idea that at warmer temperatures possums choose to eat less protein due to limitations imposed by heat.

Much of what we know about the effects of heat stress comes from domestic animal studies. In these systems chronic heat exposure shifts the use of energy substrates away from fatty acid oxidation (with the higher DIT this generates) towards glucose use (Baumgard & Rhoads, 2012). Of note, protein synthesis is reduced and protein catabolism is increased under chronic heat stress in cattle, pigs, rabbits, (Bianca, 1965; Hall *et al.*, 1980; Marder *et al.*, 1990; Wheelock *etal.*, 2010). Increased protein catabolism likely results in increased gluconeogenesis (Collins, Mitros & Skibba, 1980; Baumgard & Rhoads, 2012). However, after chronic heat exposure, blood glucose is often reduced regardless of increased intestinal absorptive capacity, hepatic output and renal resorption, indicating heat stress causes negative energy balance (Baumgard & Rhoads, 2012; Belhadj Slimen *et al.*, 2016). Again this emphasises that that ambient temperature does not simply change energy requirements, but that nutrient uptake and utilisation can also be altered by heat exposure. If animals are eating less protein in

the heat to avoid DIT as indicated by our experiment, increased protein catabolism and reduced protein synthesis are adaptive physiological responses. This also points to the main savings in terms of heat production arising from digestion. However, this also points to less available protein for growth and reproduction in wild herbivores that are reducing protein intake in response to warm ambient temperature conditions.

It is possible that other physiological factors could interplay to culminate in reduced protein intake at warmer ambient temperatures. For example, limits to the excretion of nitrogenous waste could control protein intake at high ambient temperatures through a tradeoff with water use for excretion and water use for evaporative cooling. Nitrogenous wastes are generated from both dietary protein breakdown and hepatic metabolism of body proteins from skeletal muscle which also tends to be increased by heat stress (Bianca 1965; Conte et al. 2018; Hall et al. 1980; Marder et al. 1990; Wheelock et al. 2010). In herbivores, a major mitigator of this is urea recycling to the gut, as a source of non-protein N for microbial growth. For example common brushtailed possums recycled 59% of endogenously produced urea, and produced a relatively small amount of urea compared to other herbivores on comparable diets (Foley and Hume 1987). When consuming diets low in protein, this can represent a substantial fraction of urea produced (Chilcott and Hume 1984) and the rate of urea returned to the gut depends on the rate of microbial fermentation in the gut (Reynolds and Kristensen 2008). In ruminants high ambient temperature leads to no change in renal urea excretion (a relative increase as intake is decreased) but causes a decrease in urea recycling to the gut (Obitsu et al. 2011). However the effects of ambient temperature on urea recycling have not been investigated in small hindgut fermenters such as the brushtail possum.

The diets that were offered to brushtail possums were compared to the nutritional composition of 25 *Eucalyptus melliodora* trees within the home ranges of free-living possums at Black Mountain Peninsula, Canberra, Australia (Figure 3). Young and mature leaves were collected and analyzed for available nitrogen by Marsh et al. (2018), using the same method as the current study. Unpublished data on the soluble fraction lost during the available nitrogen assay was also available for the same trees as a proxy for carbohydrate and could be compared to the soluble fraction for our available nitrogen assay. The P:NP ratios selected by possums at all three temperatures in our experiments

would be achievable for wild possums if they were to select from the young and mature leaves on the available trees on Black Mountain Peninsula (Figure 3). Our diets did not allow selection of a diet with similar macronutrient composition to unexpanded leaves, however these leaves are limited in their availability. This indicates that possums could reach the same balance of protein and non-protein energy using mostly young and mature leaves in a natural setting, giving ecological grounding to our results.

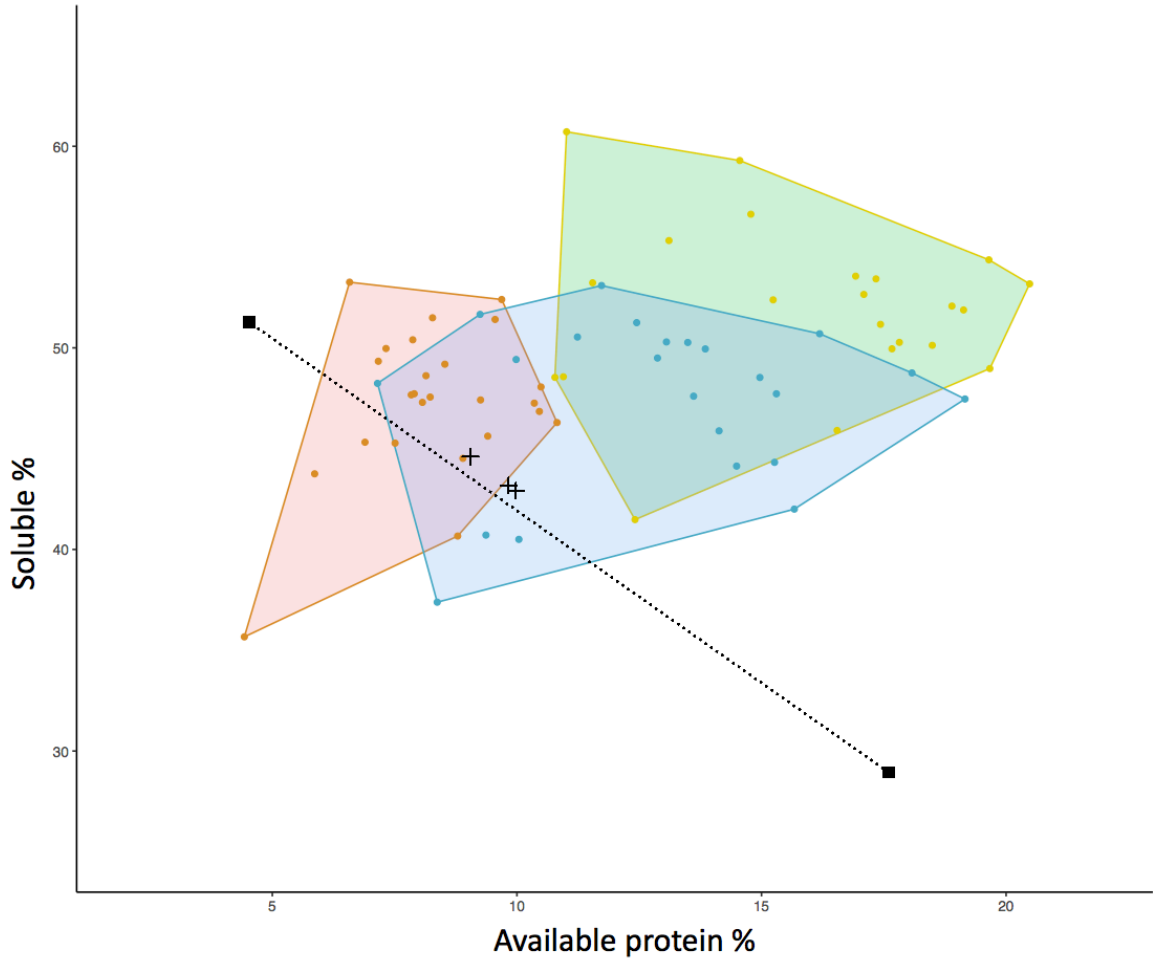

Figure 3: *The nutritional composition of diets chosen by possums after exposure to 10, 18, or 26°C for one week as compared to leaves in the home range of possums. The dotted line indicates possible compositions given the two diets (black points). The polygons indicate the food trees available within the home ranges of free-living brushtail possums. Polygon colours indicate mature leaves (orange), fully expanded young leaves (blue), and unexpanded leaves (yellow). Crosses indicate average diet composition selected at each temperature in the first experiment.*

However, possums consuming a natural diet are also contending with an array of PSMs. When the PSM 1,8-cineole was included in the diet in experiment 2, possums chose to eat less food overall, and a combination that was higher in protein than the combination of foods chosen in the cineole-free treatments. Cineole causes an increase in protein turnover in brushtail possums and is a known feeding deterrent (Au et al. 2013; Boyle and McLean 2004). The shift towards a higher protein diet compared to the cineole-free diets can be explained by the fact that possums consume more cineole when supplemented with additional protein (Au et al. 2013; Nersesian et al. 2012). What is interesting is the elimination of a temperature effect on diet composition when cinoele is added to food, even though a temperature effect on total food intake remained. Since cineole reduced intake below the level of the basal diet, the total DIT would be much lower. Meaning, when cineole was added in this experiment, while the diet chosen by possums was higher in P:NP, the absolute amount of protein ingested is much lower. Interestingly, 1,8 cineole is an agonist of TRPM8 cold receptors, which means possums ingesting 1,8 cineole may feel artificially cool. Treatment with TRPM8 agonists influences thermoregulatory behaviour, and facultative thermogenesis in rodents in a similar way to if they were exposed to cool temperatures (Jiang et al. 2017). However it remains unknown how this compound influences thermoregulation in marsupials. Therefore, while temperature may have an effect on macronutrient selection this can be altered by PSMs in the diet, potentially including the specific action of those PSMs.

We have shown that possums reduce P:NP selection with increased ambient temperature, but that PSMs can also influence the impact of temperature on P:NP selection. The diets that possums selected are within the range available in young and mature *Eucalyptus melliodora* leaves in their natural habitat. This demonstrates that the choices that possums were making are ecologically relevant. Our study, like most captive animal studies, examined the effect of constant temperatures, in this case on nutrition. However, animals in the wild are exposed to complex thermal landscapes. The ambient temperature experienced by an animal fluctuates constantly both temporally and geographically. To capture how animals respond to the complexity of a variable nutrient and thermal environment, we suggest wild animal studies as a next step. This research has expanded our view of how wild herbivores faced with both complex

nutrient landscapes and changing thermal environments might adjust their nutritional targets, or perhaps might encounter nutritional limits going forwards, and then give us insight into how these adjustments impact populations. The importance of protein for reproduction in animal populations is paramount. “Food and sex are basic for life, but of the two, food comes first. Without adequate nutrition, animals do not reproduce” (Provenza and Villalba 2006). Reduced protein intake in response to warming climates could have significant detrimental impacts on animal populations. Protein intake affects everything from spermatogenesis and ovulation (Fletcher 1981; Melo et al. 2014), to growth and survival of offspring, to timing of sexual maturation, sexual senescence and first parturition (Guzmán et al. 2006; White 1983). Protein restriction during pregnancy can also impact health later in life in progeny (Blumfield et al. 2015; Zohdi et al. 2014). These changes in reproductive performance have flow on effects to populations and often what seems life a small difference in individual nutrition can result in dramatic demographic shifts (White 1983). These “amplifier effects” mean that any change in nutrition, like those demonstrated in our study, due to warmer climates, could have significant consequences in wild herbivores.

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References

Allen ME, Ullrey DE (2004) Relationships among nutrition and reproduction and relevance for wild animals. Zoo Biol 23:475-487. doi: 10.1002/zoo.20029

Au J, Marsh KJ, Wallis IR, Foley WJ (2013) Whole-body protein turnover reveals the cost of detoxification of secondary metabolites in a vertebrate browser. J Comp Physiol B 183:993-1003

Baumgard LH, Rhoads RP (2012) Ruminant nutrition symposium: Ruminant production and metabolic responses to heat stress. J Anim Sci 90:1855-1865

Baumgard LH, Wheelock JB, Sanders SR, Moore CE, Green HB, Waldron MR, Rhoads RP (2011) Postabsorptive carbohydrate adaptations to heat stress and monensin supplementation in lactating Holstein cows. J Dairy Sci 94:5620-5633

Beale PK, Marsh KJ, Foley WJ, Moore BD (2017) A hot lunch for herbivores: physiological effects of elevated temperatures on mammalian feeding ecology. Biol Rev Camb Philos Soc. doi: 10.1111/brv.12364

Bianca W (1965) Reviews of progress of dairy science: Section A. Physiology. Cattle in a hot environment. J Dairy Res 32:291-328

Blumfield ML, Nowson C, Hure AJ, Smith R, Simpson SJ, Raubenheimer D, MacDonald-Wicks L, Collins CE (2015) Lower Protein-to-Carbohydrate Ratio in Maternal Diet is Associated with Higher Childhood Systolic Blood Pressure up to Age Four Years. Nutrients 7:3078-3093. doi: 10.3390/nu7053078

Boyle RR, McLean S (2004) Constraint of feeding by chronic ingestion of 1,8-cineole in the brushtail possum (*Trichosurus vulpecula*). J Chem Ecol 30:757-775

Camp MJ, Shipley LA, Milling CR, Rachlow JL, Forbey JS (2018) Interacting effects of ambient temperature and food quality on the foraging ecology of small mammalian herbivores. J Therm Biol 71:83-90. doi: https://doi.org/10.1016/j.jtherbio.2017.10.021

Chilcott MJ, Hume ID (1984) Nitrogen and urea metabolism and nitrogen requirements of the common ringtail possum, *Pseudocheirus peregrinus*, fed *Eucalyptus andrewsii* foliage. Aust J Zool 32:615-622

Conte G, Ciampolini R, Cassandro M, Lasagna E, Calamari L, Bernabucci U, Abeni F (2018) Feeding and nutrition management of heat-stressed dairy ruminants. Italian Journal of Animal Science 17:604-620. doi: 10.1080/1828051X.2017.1404944

Dawson TJ (1969) Temperature regulation and evaporative water loss in the brush-tailed possum *Trichosurus vulpecula*. Comp Biochem Physiol 28:401-407

Dearing MD (2013) Temperature-dependent toxicity in mammals with implications for herbivores: a review. J Comp Physiol B 183:43-50

Dearing MD, Foley WJ, McLean S (2005) The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. Annu Rev Ecol Evol Syst 36:169-189

DeGabriel JL, Moore BD, Foley WJ, Johnson CN (2009) The effects of plant defensive chemistry on nutrient availability predict reproductive success in a mammal. Ecology 90:711-719

Felton AM, Felton A, Raubenheimer D, Simpson SJ, Krizsan SJ, Hedwall P-O, Stolter C (2016) The nutritional balancing act of a large herbivore: an experiment with captive moose (*Alces alces* L). PLoS. ONE 11:e0150870. doi: 10.1371/journal.pone.0150870

Ferguson NS, Gous RM (1997) The influence of heat production on voluntary food intake in growing pigs given protein-deficient diets. Animal Science 64:365-378. doi: 10.1017/S1357729800015939

Fletcher I (1981) Effects of energy and protein intake on ovulation rate associated with the feeding of lupin grain to Merino ewes. Aust J Agric Res 32:79-87. doi: 10.1071/AR9810079

Foley WJ, Hume ID (1987) Nitrogen Requirements and Urea Metabolism in Two Arboreal Marsupials, the Greater Glider (*Petauroides volans*) and the Brushtail Possum (*Trichosurus vulpecula*), Fed Eucalyptus Foliage. Physiol Zool 60:241-250

Foley WJ, Iason G, McArthur C (1999) Role of plant secondary metabolites in the nutritional ecology of mammalian herbivores - how far have we come in 25 years? In: Jung H-JG, Fahey GCJ (eds) Vth International Symposium on the Nutrition of Herbivores. American Society of Animal Science, Savoy, pp 203-274

Forbey JS, Foley WJ (2009) PharmEcology: A pharmacological approach to understanding plant-herbivore interactions. Integr Comp Biol 49:267-273

Guzmán C, Cabrera R, Cárdenas M, Larrea F, Nathanielsz PW, Zambrano E (2006) Protein restriction during fetal and neonatal development in the rat alters reproductive function and accelerates reproductive ageing in female progeny. The Journal of Physiology 572:97-108. doi: 10.1113/jphysiol.2005.103903

Hall GM, Lucke JN, Lovell R, Lister D (1980) Porcine malignant hyperthermia. VII: Hepatic metabolism. British Journal of Anaesthesia 52:11-17. doi: 10.1093/Bja/52.1.11

Hammond K, Diamond J (1994) Limits to dietary nutrient intake and intestinal nutrient uptake in lactating mice. Physiol Zool 67:282-303

Iason G (2005) The role of plant secondary metabolites in mammalian herbivory: Ecological perspectives. Proc. Nutr. Soc 64:123-131

Jiang C, Zhai M, Yan D, Li D, Li C, Zhang Y, Xiao L, Xiong D, Deng Q, Sun W (2017) Dietary menthol-induced TRPM8 activation enhances WAT "browning" and ameliorates diet-induced obesity. Oncotarget 8:75114-75126. doi: 10.18632/oncotarget.20540

Kurnath P, Dearing MD (2013) Warmer ambient temperatures depress liver function in a mammalian herbivore. Biol Lett 9:20130562. doi: 10.1098/rsbl.2013.0562

Kurnath P, Merz ND, Dearing MD (2016) Ambient temperature influences tolerance to plant secondary compounds in a mammalian herbivore. Proc R Soc Lond B 283:20152387

Marder J, Eylath U, Moskovitz E, Sharir R (1990) The effect of heat exposure on blood chemistry of the hyperthermic rabbit. Comparative Biochemistry and Physiology A-Physiology 97:245-247. doi: 10.1016/0300-9629(90)90179-V

Marsh KJ, Ward J, Wallis IR, Foley WJ (2018) Intraspecific variation in nutritional composition affects the leaf age preferences of a mammalian herbivore. J Chem Ecol 44:62-71. doi: 10.1007/s10886-017-0911-3

McArt SH, Spalinger DE, Collins WB, Schoen ER, Stevenson T, Bucho M (2009) Summer dietary nitrogen availability as a potential bottom-up constraint on moose in south-central Alaska. Ecology 90:1400-1411

Melo MC, Almeida FRCL, Caldeira-Brant AL, Parreira GG, Chiarini-Garcia H (2014) Spermatogenesis recovery in protein-restricted rats subjected to a normal protein diet after weaning. Reprod Fertil Dev 26:787-796. doi: 10.1071/RD13032

Nersesian CL, Banks PB, Simpson SJ, McArthur C (2012) Mixing nutrients mitigates the intake constraints of a plant toxin in a generalist herbivore. Behav Ecol 23:879-888. doi: 10.1093/beheco/ars049

Obitsu T, Kamiya M, Kamiya Y, Tanaka M, Sugino T, Taniguchi K (2011) Effects of high ambient temperature on urea-nitrogen recycling in lactating dairy cows. Anim. Sci. J. 82:531-536. doi: 10.1111/j.1740-0929.2011.00880.x

Pahl L (1987) Survival, age-determination and population age structure of the common ringtail possum, *Pseudocheirus peregrinus*, in a *Eucalyptus* woodland and a *Leptospermum* thicket in southern Victoria. Aust J Zool 35:625-639. doi: 10.1071/ZO9870625

Provenza FD, Villalba JJ (2006) Foraging in domestic herbivores: linking the internal and external milieux. In: Bels VL (ed) Feeding in Domestic Vertebrates: From Structure to Behavior. CABI Publishing, Oxfordshire, UK, pp 210 - 240

Renaudeau D, Collin A, Yahav S, de Basilio V, Gourdine JL, Collier RJ (2012) Adaptation to hot climate and strategies to alleviate heat stress in livestock production. Animal 6:707-728. doi: 10.1017/S1751731111002448

Reynolds CK, Kristensen NB (2008) Nitrogen recycling through the gut and the nitrogen economy of ruminants: an asynchronous symbiosis. J. Anim. Sci. 86:E293-305. doi: 10.2527/jas.2007-0475

Rhoads RP, Baumgard LH, Suagee JK, Sanders SR (2013) Nutritional interventions to alleviate the negative consequences of heat stress. Advances in Nutrition 4:267-276

Scholander PF (1955) Evolution of climatic adaptation in homeotherms. Evolution 9:15-26

Secor SM (2009) Specific dynamic action: a review of the postprandial metabolic response. J Comp Physiol B 179:1-56

Simpson SJ, Raubenheimer D (2001) The geometric analysis of nutrient-allelochemical interactions: A case study using locusts. Ecology 82:422-439

Villalba JJ, Provenza FD (2005) Foraging in chemically diverse environments: Energy, protein, and alternative foods influence ingestion of plant secondary metabolites by lambs. J Chem Ecol 31:123-138. doi: 10.1007/s10886-005-0979-z

Villalba JJ, Provenza FD, Banner RE (2002a) Influence of macronutrients and activated charcoal on intake of sagebrush by sheep and goats. J. Anim. Sci. 80:2099-2109

Villalba JJ, Provenza FD, Banner RE (2002b) Influence of macronutrients and polyethylene glycol on intake of a quebracho tannin diet by sheep and goats. J. Anim. Sci. 80:3154-3164

Villalba JJ, Provenza FD, Bryant JP (2002c) Consequences of the interaction between nutrients and plant secondary metabolites on herbivore selectivity: Benefits or detriments for plants? Oikos 97:282-292

West JW (1999) Nutritional strategies for managing the heat-stressed dairy cow. J Anim Sci 77:21-35

Westerterp KR (2004) Diet-induced thermogenesis. Nutrition and Metabolism 1:5-5

Wheelock JB, Rhoads RP, VanBaale MJ, Sanders SR, Baumgard LH (2010) Effects of heat stress on energetic metabolism in lactating Holstein cows. J Dairy Sci 93:644-655

White RG (1983) Foraging patterns and their multiplier effects on productivity of northern ungulates. Oikos 40:377-384. doi: 10.2307/3544310

Windley HR, Wallis IR, DeGabriel JL, Moore BD, Johnson CN, Foley WJ (2013) A faecal index of diet quality that predicts reproductive success in a marsupial folivore. Oecologia 173:203-212

Winter J (1980) Tooth wear as an age index in a population of the brush-tailed possum, *Trichosurus vulpecula* (Kerr). Wildl Res 7:359-363. doi: 10.1071/WR9800359

Zohdi V, Lim K, Pearson JT, Black MJ (2014) Developmental programming of cardiovascular disease following intrauterine growth restriction: findings utilising a rat model of maternal protein restriction. Nutrients 7:119-152. doi: 10.3390/nu7010119

Chapter 5

Daily variation in ambient temperatures affect the fine scale feeding decisions of wild koalas

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Abstract

The feeding decisions of mammalian herbivores had, until recently, been considered intrinsic to the food and the animal, influenced by, for example, the relative concentrations of particular PSMs and nutrients in the food, previous experience of the animal, and the capacity of the animal to detoxify PSMS and to excrete the metabolites. However, captive studies in several species have now shown that an extrinsic factor, ambient temperature, also alters PSM tolerance and food choice. Although captive studies are critical for describing the relationship between ambient temperature and feeding under controlled conditions, ultimately, the goal is to understand whether fine-scale daily fluctuations in ambient temperature influence the feeding decisions of wild herbivores. In order to address this question, we used data from a previous study that examined the feeding choices of semi-wild koalas (*Phascolarctos cinereus*), and extended the analyses to consider the impact of ambient temperature. We found that koalas reduced their daily food intake at warmer mean ambient temperatures (approximately 6g decrease in dry matter intake per 1°C increase in mean daily temperature). This was due to smaller meal sizes without an increase in meal frequency. At warmer temperatures, koalas particularly reduced the size of meals when the concentration of one group of PSMs, the formylated phloroglucinol compounds (FPCs), in foliage was high. There was a positive relationship between the foliar concentrations of FPCs and available nitrogen (N), which meant that the intake of available N by koalas depended on both the ambient temperature and the FPC concentration of leaves. These results demonstrate that daily fluctuations in ambient temperatures impact the feeding decisions of wild herbivores, and that they are likely

to eat less in the future as the climate warms. Furthermore, these effects are likely to be exacerbated when herbivores ingest plants containing high concentrations of PSMs.

Introduction

Browsing mammals must meet their nutrient requirements without over-ingesting potentially toxic plant secondary metabolites (PSMs) that are present in most tree and shrub foliage (Dearing, Foley & McLean 2005). The food preferences of mammalian herbivores are a response to intrinsic qualities of the food (e.g. nutrient and PSM concentrations, Dearing, Foley & McLean 2005; Felton *et al.* 2009; Forbey *et al.* 2013), the animal's physiology (e.g. nutritional state, and capacity to metabolise and excrete PSMs, Dearing, Skopec & Bastiani 2006; Skopec, Haley & Dearing 2007; Torregrossa & Dearing 2009; Kohl *et al.* 2014) and past experience (e.g. previous physiological feedback after ingesting the food, Provenza *et al.* 2003). However, studies in several species have now shown that an extrinsic factor, ambient temperature, also alters food intake, diet selection, and tolerance to PSMs (Chapters 2-5, Dearing *et al.* 2008; Kurnath, Merz & Dearing 2016).

Herbivores in agricultural systems often dramatically reduce food intake at warmer ambient temperatures (e.g. Close, Mount & Start 1971; Bernabucci *et al.* 1999; Ominski *et al.* 2002; Renaudeau, Quiniou & Dubois 2002), probably because the digestion of food produces heat, and there are limitations on the rate at which animals can dissipate heat at warm temperatures (Speakman & Król 2010; Beale *et al.* 2018). This is often accompanied by reduced productivity as a consequence of reduced nutrient intake (e.g. Ominski *et al.* 2002; Rhoads *et al.* 2009). Further, ambient temperature can impact macronutrient selection by herbivores. In cooler seasons, golden snub-nosed monkeys met additional energy requirements by eating more non-protein energy (Guo *et al.* 2018). In captive feeding studies, common brushtail possums (*Trichosurus vulpecula*) chose a diet lower in protein at higher ambient temperatures compared to cool when

the diet did not include PSMs (Chapter 4). However, when the diet contained PSMs, possums selected a diet higher in protein than the one they selected in the absence of PSMs, but also ate less at warmer temperatures than at cool (Chapter 4). In fact, several laboratory-based studies have indicated temperature-dependent effects on herbivores ingesting PSM-rich diets. For example, woodrats (genus *Neotoma*) were able to persist on PSM-rich and PSM-free diets equally well when housed at cool temperatures, but were unable to persist on PSM-rich diets when housed at warm temperatures (Kurnath, Merz & Dearing 2016). Likewise, the marsupial folivores, common brushtail and common ringtail possums (*Pseudocheirus peregrinus*) ate less of both PSM-free and PSM-containing diets when exposed to warm ambient temperatures for one week compared to cool temperatures (Chapter 3). Together, these studies demonstrate that changes in ambient temperature can alter the food intake, diet selection and PSM tolerance of herbivores, and that at warmer temperatures, these effects may be detrimental to productivity.

Koalas (*Phascolarctos cinereus*) are the most iconic of the marsupial folivores. They ingest a highly-restricted diet of *Eucalyptus* foliage, which can vary in nutrient and PSM concentrations both within and between eucalypt species (Moore *et al.* 2005). One particular class of PSMs, the formylated phloroglucinol compounds (FPCs) are important deterrents for a number of marsupial and insect herbivores . These PSMs also influence tree selection by, and distribution and abundance of koalas (Norton & Neave 1990; Moore *et al.* 2010; Au 2018). Koalas generally obtain water solely through their diet, but koalas have been observed descending from trees and searching for drinking water during heat waves in eastern Australia. Likewise, there have been increased reports of heat stress in koalas in recent years, and hyperthermia has been blamed for drastic

population reductions (Gordon, Brown & Pulsford, 1988*b*; Lunney *et al.*, 2012). We propose that these effects could be exacerbated if high ambient temperatures impose constraints on food (and therefore foliar water) intake by koalas, and that intake limitations may be most severe when koalas feed from trees with high FPC concentrations.

Here we investigate how ambient temperature influences food intake and selection by semi-wild koalas using data collected from an earlier study of koalas living freely within a 7.6 ha enclosure of natural forest on Phillip Island, Victoria, Australia (Marsh *et al.* 2014b). Six animals were each continuously monitored for a two-week period using a combination of radio- and audio-telemetry to record the location and duration of all meals (Marsh *et al.* 2014b). The chemical composition (available nitrogen (N) and FPC concentrations) of the leaves eaten at each meal was also measured (Marsh *et al.* 2014b). Available N is an *in vitro* estimate of the effect of tannins on protein digestibility and is always less than the total N concentration of the same sample (Wallis, Nicolle & Foley 2010). Data on ambient temperature was collected at the site, but was not used in the original analysis of koala feeding behaviour. Based on observations from captive and domestic herbivores, we predicted that koalas would eat less on days on which the mean ambient temperature was higher. Further to this, we investigated whether the difference in food intake was due to a change in the number of meals eaten per day, or in the size of individual meals. To better understand whether natural daily fluctuations in ambient temperatures affect feeding behaviour at a fine scale, we also investigated whether interactions between ambient temperature and nutritional composition (available N and FPC concentration) affected the size of individual meals eaten by koalas. Finally, because both ambient temperature and dietary PSMs have previously

been shown to influence the intake of protein by herbivores, we investigated whether ambient temperature and the foliar FPC concentration affected the per meal intake of available N by koalas. We expected that available N intake would be reduced with increasing ambient temperature, and that this effect would be exacerbated if koalas were feeding in trees with high concentrations of FPCs.

Methods

The methods for monitoring feeding by koalas and analyzing leaf nutritional composition are described in detail in (Marsh *et al.* 2014b). In that study, eight koalas were monitored two at a time for two weeks each between February and May, 2004. However, temperature data was only collected during the monitoring periods of six koalas, so the current analysis is restricted to those six individuals.

Dry matter intake (DMI) of each meal was estimated by the relationship between food intake (g dry matter) and meal length (mins) in six captive koalas fed *Eucalyptus viminalis* foliage from 18 different trees during 550 meals (Marsh, Wallis & Foley 2007). Daily intake was calculated as the sum of DMI for each meal over a 24h period beginning at 1500 h. The final relationship between food intake and meal length is given by the following equation:

$$\text{Dry matter intake} = (1.2144 \times \text{meal length}) - 1.9022$$

The ambient temperature was recorded at hourly intervals throughout the study using a calibrated thermocouple attached to a datalogger. The datalogger was placed in a weatherproof box in a shaded position at a central location within the reserve.

All data was analysed by generalized linear mixed models in R (GLMM, packages: lme4, lmerTest). In order to understand whether ambient temperature influenced the daily food intake of semi-wild koalas, we examined whether mean ambient temperature within a day (starting at 1500 h to coincide with the period of lowest activity by koalas, Marsh *et al.* 2014a) influenced the total food intake within that day (daily DMI). The model included daily DMI as the dependent variable, mean temperature as the independent variable, and koala identity and day as random variables. Following this, we examined whether mean temperature in a day influenced the number of meals by including number of meals as the dependent variable, mean temperature as the independent variable, and koala identity and day as random variables. We then examined whether mean temperature in a day influenced the daily available N intake by including daily available N intake as the dependent variable, mean temperature as the independent variable, and koala identity and day as random variables.

We examined whether the nutritional composition (FPC and available N concentration) of leaves and the ambient temperature at the time of the meal influenced either intake on a per meal basis (per meal DMI) or intake of available N using two GLMMs. The first model had per meal DMI as the dependent variable, temperature, FPC concentration and available N concentrations as continuous independent variables with an ambient temperature x FPC concentration interaction, and koala identity and a term indicating which order each meal occurred within the day as random variables. In the second model, the dependent variable was replaced with the available N intake per meal and available N concentration was not used as an independent variable.

We also tested the relationship between foliar FPC concentration and available N concentration in each meal. The GLMM included available N concentration as the

dependent variable and FPC concentration as the independent variable with koala identity and meal order as random variables.

Results

Koalas reduced their daily DMI with increasing mean ambient temperature ($t = -2.513$, $P = 0.01$; Figure 1). However, there was no effect of mean ambient temperature on the number of meals within a day ($t = -0.844$, $P = 0.401$), meaning that the reduction in daily DMI was due to a change in meal size, not in meal frequency. There was also a near-significant trend for daily available N intake to be reduced by increased mean temperature ($t = -2.044$, $P = 0.054$)

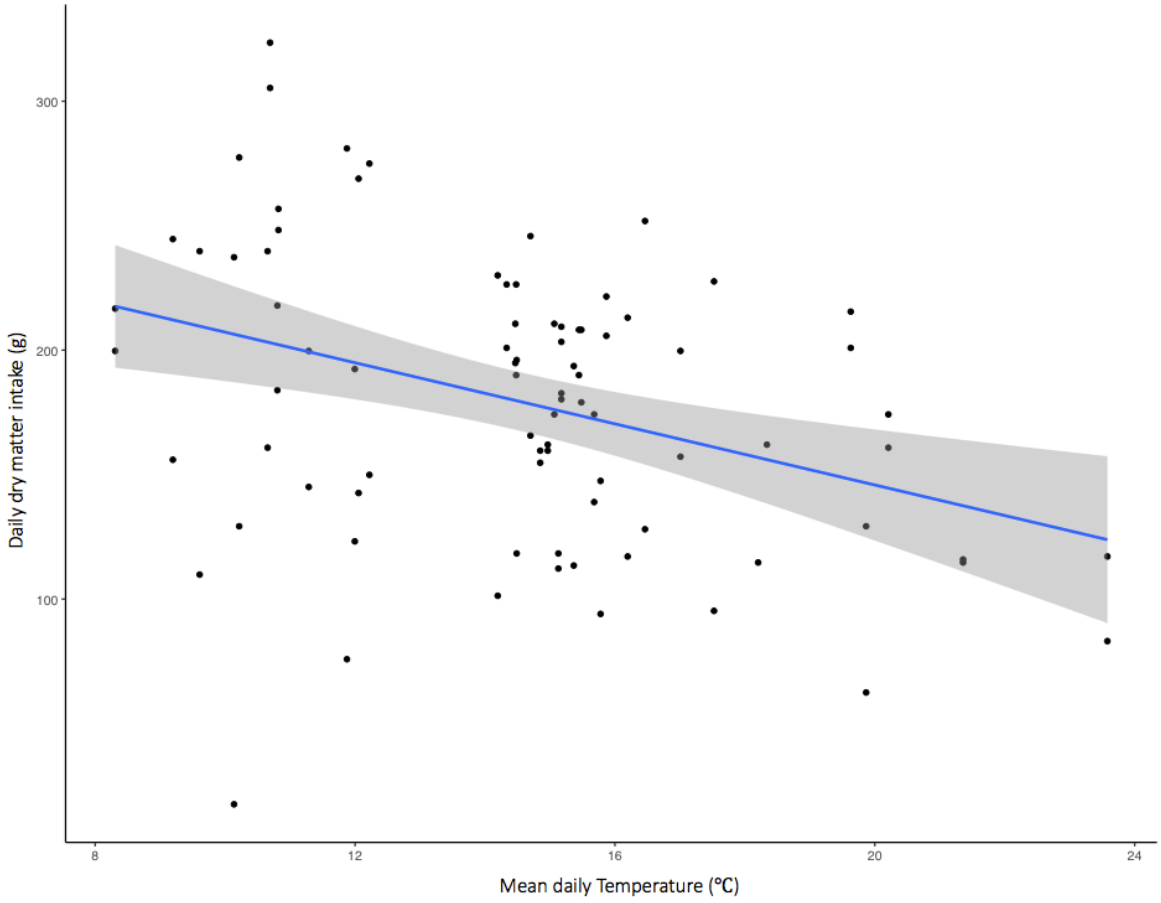

Figure 1. The relationship between mean ambient temperature within a day and total dry matter intake for that day. The line shows the fitted model from the statistical analysis, with the grey ribbon indicating the standard error.

There was an overall trend for koalas to reduce per meal DMI at increasing temperatures (t = 1.739, P = 0.08), while higher FPC concentrations corresponded with lower per meal DMI (t = 2.958, P = 0.003). The available N concentration did not influence per meal DMI (t = 0.365, P = 0.715). There was a significant interaction between the foliar FPC concentration of trees from which koalas chose to feed and ambient temperature, such that per meal DMI from trees with higher concentrations of FPCs was reduced by warmer ambient temperatures to a greater extent than was the per meal DMI from trees with lower FPC concentrations (t = -3.055, P = 0.002; Figure 2).

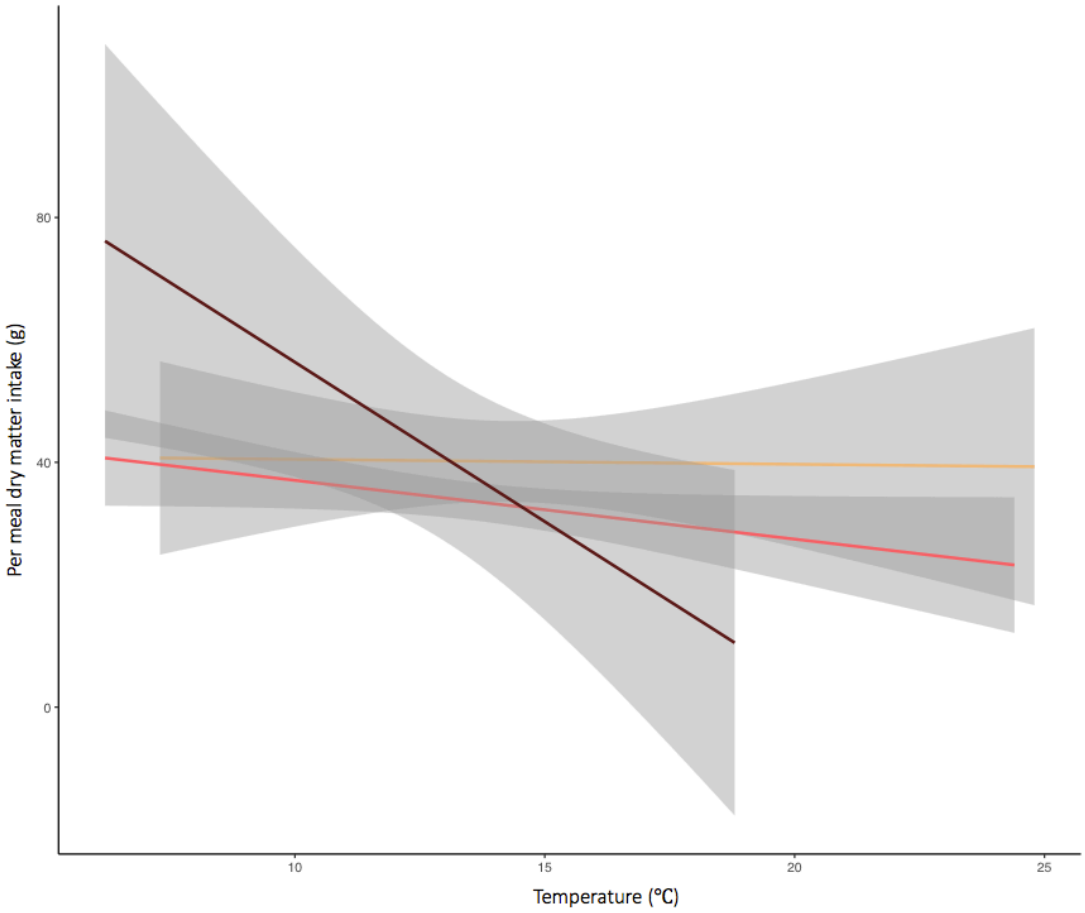

***Figure 2**. Liner model predictions for ambient temperature effects on per meal DMI by koalas within three categories of FPC concentration, low (yellow, 0-15 mg.g^{-1} DM), mid (red, 15-30 mg.g^{-1} DM), and high (brown, >30 mg.g^{-1} DM). Note that there were no meals from trees in the "high" FPC category above 18˚C. The grey ribbons indicate the standard error.*

Trees with higher FPC concentrations also had higher available N concentrations (t = 9.791 P < 0.001). Intake of available N by koalas increased with ambient temperature (t = 2.632 P = 0.009), and with the FPC concentration of the foliage (t = 4.514 P < 0.001). However, there was a significant interaction between ambient temperature and FPC concentration such that higher FPC concentrations led to lower available N intakes at warmer temperatures than at cooler temperatures, while at lower FPC concentrations available N intake increased at warmer ambient temperatures (t = -3.798 P < 0.001).

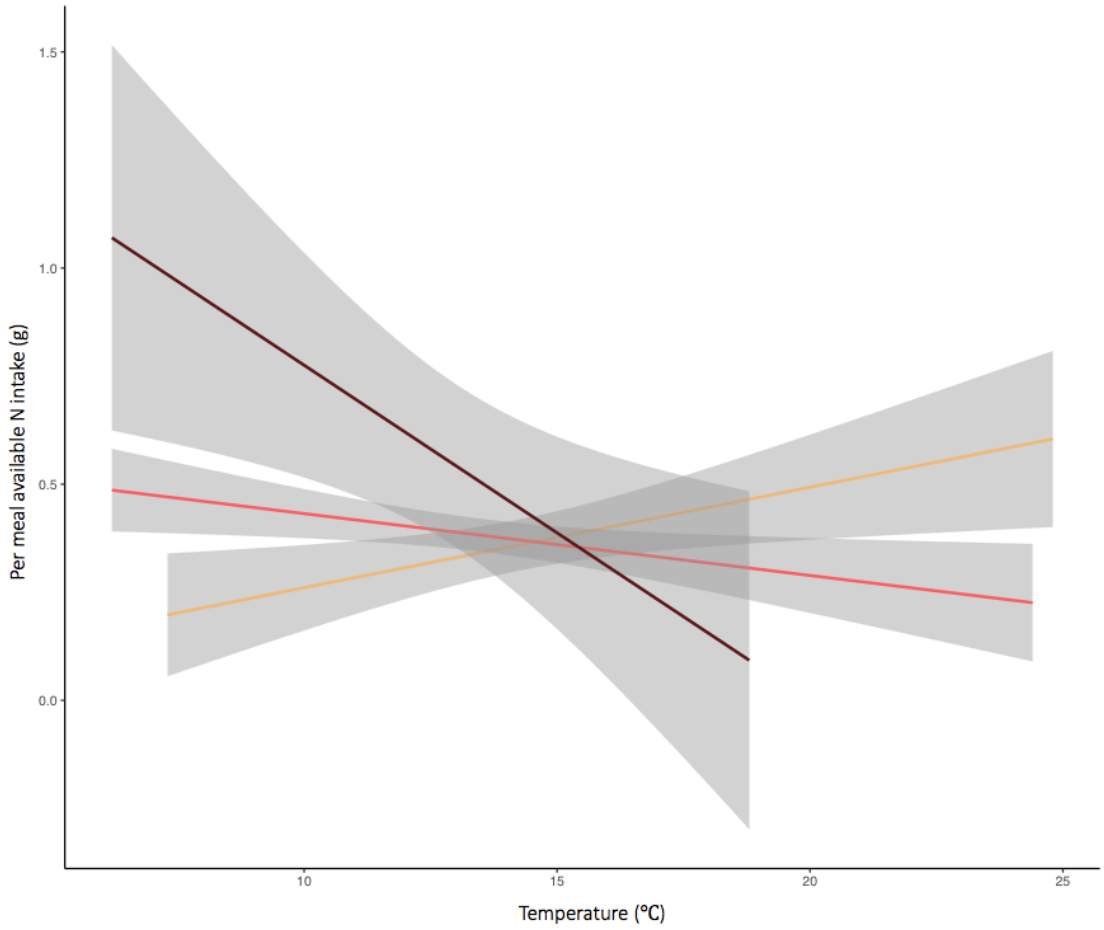

Figure 3. *Linear model predictions of per meal available N intake across different ambient temperatures within categories of FPC concentration, low (yellow, 0-15 mg.g^{-1} DM), mid (red, 15-30 mg.g^{-1} DM), and high (brown, >30 mg.g^{-1} DM). Note that there were no meals from trees in the "high" FPC category above 18˚C. The grey ribbons indicate the standard error.*

Discussion

We have demonstrated that on warm days free-ranging koalas eat less (Figure 1), and that this reduction in food intake is due to reduced meal size. In particular, warmer temperatures depress per meal DMI when koalas are feeding in trees with high foliar concentrations of FPCs (Figure 2). Of the trees which koalas chose for meals, foliage

with higher FPC concentrations also had higher available N concentrations. This meant that at cooler temperatures koalas were able to choose leaves with higher concentrations of FPCs and access more protein, but at warmer temperatures koalas did not select leaves with high FPC concentrations (Figure 3). Extended periods of warm temperatures may compound these effects by reducing food and available N intake over a longer period.

Disentangling the effects of temperature on energy requirements, on food or nutrient intake, and on PSM tolerance is difficult. For example, it is expected that warm temperatures depress food intake and, as a result, intake of specific foliar constituents would also decline, unless animals alter their selection to favour foods with higher concentrations of those constituents. Likewise, many other factors contribute to diet selection in wild herbivores aside from the contents of potential food, such as social interactions or predator avoidance (Seabloom & Reichman 2001; McArthur *et al.* 2014). For these reasons, previous investigations of the effects of temperature on feeding by herbivores have predominantly been conducted in controlled laboratory settings (e.g. Chapters 3-4, Kurnath, Merz & Dearing 2016). Most laboratory studies are conducted under constant temperature regimes that miss the influence of daily temperature fluctuations, or fluctuations over longer timescales. But our results suggest that animals respond quickly to changes in temperature, and so the temporality of fluctuations may be important. Captive studies are also typically no-choice experiments, measuring intake of a single diet on offer, which, while valuable, do not capture the complexity faced by a wild herbivore surrounded by plants containing various mixtures of both chemicals and nutrients. Thus, this study is the first to show that temperature-dependent effects on food intake, available protein intake, food choice, and PSM

tolerance are all detectable in free-ranging herbivores consuming their natural diet. Perhaps our results will stimulate reexamination of data collected in other plant-herbivore systems.

Our results suggest that koalas are less tolerant of trees with high FPC concentrations at high ambient temperatures than they are at lower temperatures. They did not feed from any trees in the "high" FPC category (FPC concentration > 30 $mg.g^{-1}$ DM) at temperatures above 18 °C, and they reduced per meal DMI of trees in this category at a greater rate compared to trees in other categories in response to increasing ambient temperature. One reason that herbivores may be less tolerant of PSMs as ambient temperature increases is temperature dependent toxicity (TDT). At least one mechanism responsible for TDT is thought to be a reduced rate of metabolism of PSMs at warm ambient temperatures (Chapter 2, Kurnath & Dearing 2013). The purported reason for this down-regulation of detoxification capacity when experiencing warmer ambient temperatures, is an inability to dissipate the excess heat that the detoxification processes generate (Dearing 2013; Beale *et al.* 2018). It is not clear whether TDT is operating in koalas feeding on *Eucalyptus* foliage, or whether there are other limitations that restrict FPC intake at high temperatures (such as increased time in the gut prior to absorption). This could be an area to investigate in the future. However, the results support experiments with ringtail possums exposed to warm temperatures, where animals consumed less when offered *Eucalyptus melliodora* leaves that contained high FPC concentrations compared to low FPC concentrations (chapter 3), and also showed reduced hepatic detoxification capacities (chapter 2).

One of the other consequences of koalas reducing daily DMI (Figure 3) at higher ambient temperatures was the trend to reduce available N intake. This was due to

eating less (Figure 1), but also because they chose trees with lower FPC concentrations (Figure 2). We had predicted that feeding from higher FPC trees would cause a more dramatic reduction in available N intake with warmer temperatures. However, since FPC concentration and available N concentration were tightly correlated in the trees from which koalas fed, they would have had to eat trees with high FPC concentrations at high temperatures to keep available N intake stable while reducing DMI. This reduced intake of available N at warmer temperatures could pose a challenge for animal populations since adequate protein intake is essential for reproduction (Provenza & Villalba 2006), and has been linked with reproductive success in other wild herbivore species (White 1983; DeGabriel *et al.* 2009).

Here we have presented a fine scale measurement of food intake and FPC tolerance by wild koalas, and related it to the ambient temperature at the time of that meal, or the average temperature during the day. However, there are other scales at which both food intake and ambient temperature could be measured. Ambient temperature undergoes daily fluctuations and it is possible that the minimum and maximum temperatures within a day, or the time above certain threshold temperatures, may be important for influencing food selection. For example, animals may store heat for dissipation at cooler temperatures. Likewise, longer exposures to warmer ambient temperatures, could have a compounding effect by reducing cumulative food intake or by further reducing PSM tolerance. For example, in brushtail and ringtail possums, reduced PSM intake was observed following week-long exposure to warm ambient temperatures (26°C), while no difference was observed following overnight exposure (chapter 3). However, in those experiments, animals were housed at constant temperatures, animals were not offered a choice of food, and the food intake was

measured daily (chapter 3). This means that any effect of temperature fluctuations, of food choice, or any difference between individual meals within the one day was lost. Building our understanding of both immediate and long-term effects of increased temperatures through further captive and field studies would help us to predict the outcome of different climatic events on koalas.

Our results suggest that increasing ambient temperatures due to climate change have implications for the habitat requirements of koalas and other mammalian herbivores. For example, in order for an individual koala to choose to eat lower PSM trees, these trees need to be available within its home range. Likewise, if food intake is reduced with warmer ambient temperatures, less energy and nutrients will be available to animals, unless they can compensate by selecting foods with higher proportions of these nutrients. Lower intakes of available protein in particular can have detrimental long-term effects on reproduction, and hence on populations. For example, hispid cotton rats experience first oestrus when 50% older if fed diets with ecologically relevant low concentrations of protein (Eshelman & Cameron 1996), and the timing of snow melt has pronounced effect on reindeer populations due to changes in protein concentration in forage (White 1983; Cebrian, Kielland & Finstad 2008). To add further concern, trees are expected to increase in PSM and fibre concentrations with increased $CO_{2,}$ and decrease in N concentrations (Rothman *et al.* 2015; Craine *et al.* 2018), meaning as koala tolerance for PSMs declines, PSM concentrations will increase, further restricting food availability. An additional implication of this discovery, is that when considering areas for conservation purposes, not only must the available trees meet the needs of koalas today, but they must also be able to sustain koalas into the future. Given that koala food choice changes with temperature, it is likely so will the characteristics of

forests required to conserve them. Since both warmer average temperatures, and more frequent and more severe heat waves are predicted across the range of the koala (CSIRO & BOM 2015), it is imperative we understand any impact of temperature on nutrition and diet selection. Our results suggest that koalas will require habitats with abundant trees that contain low concentrations of FPCs in order to combat the intake-reducing effects of elevated temperatures in future climates.

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References.

Au, J. (2018) Multi-scale effects of nutrition on an arboreal folivore. PhD Thesis, Australian National University.

Beale, P.K., Marsh, K.J., Foley, W.J. & Moore, B.D. (2018) A hot lunch for herbivores: physiological effects of elevated temperatures on mammalian feeding ecology. *Biological Reviews of the Cambridge Philosophical Society,* **93,** 674-692.

Bernabucci, U., Bani, P., Ronchi, B., Lacetera, N. & Nardone, A. (1999) Influence of short- and long-term exposure to a hot environment on rumen passage rate and diet digestibility by Friesian heifers. *Journal of Dairy Science,* **82,** 967-973.

Cebrian, M.R., Kielland, K. & Finstad, G. (2008) Forage quality and reindeer productivity: multiplier effects amplified by climate change *Arctic, Antarctic, and Alpine Research,* **40,** 48-54.

Close, W.H., Mount, L.E. & Start, I.B. (1971) The influence of environmental temperature and plane of nutrition on heat losses from groups of growing pigs. *Animal Production,* **13,** 285-294.

Craine, J.M., Elmore, A.J., Wang, L., Aranibar, J., Bauters, M., Boeckx, P., Crowley, B.E., Dawes, M.A., Delzon, S., Fajardo, A., Fang, Y., Fujiyoshi, L., Gray, A., Guerrieri, R., Gundale, M.J., Hawke, D.J., Hietz, P., Jonard, M., Kearsley, E., Kenzo, T., Makarov, M., Marañón-Jiménez, S., McGlynn, T.P., McNeil, B.E., Mosher, S.G., Nelson, D.M., Peri, P.L., Roggy, J.C., Sanders-DeMott, R., Song, M., Szpak, P., Templer, P.H., Van der Colff, D., Werner, C., Xu, X., Yang, Y., Yu, G. & Zmudczyńska-Skarbek, K. (2018) Isotopic evidence for oligotrophication of terrestrial ecosystems. *Nature Ecology & Evolution,* **2,** 1735-1744.

CSIRO & BOM (2015) Climate change in Australia technical report. *Climate change in Australia information for Australia's natural resource management regions: technical report* (ed. P. Whetton). CSIRO and Bureau of Meteorology, Australia.

Dearing, M.D. (2013) Temperature-dependent toxicity in mammals with implications for herbivores: a review. *Journal of Comparative Physiology B,* **183,** 43-50.

Dearing, M.D., Foley, W.J. & McLean, S. (2005) The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. *Annual Review of Ecology Evolution and Systematics,* **36,** 169-189.

Dearing, M.D., Forbey, J.S., McLister, J.D. & Santos, L. (2008) Ambient temperature influences diet selection and physiology of an herbivorous mammal, *Neotoma albigula*. *Physiological and Biochemical Zoology,* **81,** 891-897.

Dearing, M.D., Skopec, M.M. & Bastiani, M.J. (2006) Detoxification rates of wild herbivorous woodrats (*Neotoma*). *Comp Biochem Physiol A Mol Integr Physiol,* **145,** 419-422.

DeGabriel, J.L., Moore, B.D., Foley, W.J. & Johnson, C.N. (2009) The effects of plant defensive chemistry on nutrient availability predict reproductive success in a mammal. *Ecology,* **90,** 711-719.

Eshelman, B.D. & Cameron, G.N. (1996) Growth and reproduction of hispid cotton rats (*Sigmodon hispidus*) in response to naturally occurring levels of dietary protein. *Journal of Mammalogy,* **77,** 220-231.

Felton, A.M., Felton, A., Raubenheimer, D., Simpson, S.J., Foley, W.J., Wood, J.T., Wallis, I.R. & Lindenmayer, D.B. (2009) Protein content of diets dictates the daily energy intake of a free-ranging primate. *Behavioral Ecology,* **20,** 685-690.

Forbey, J.S., Dearing, M.D., Gross, E.M., Orians, C.M., Sotka, E.E. & Foley, W.J. (2013) A pharm-ecological perspective of terrestrial and aquatic plant-herbivore interactions. *Journal of Chemical Ecology,* **39,** 465-480.

Guo, S.-T., Hou, R., Garber, P.A., Raubenheimer, D., Righini, N., Ji, W.-H., Jay, O., He, S.-J., Wu, F., Li, F.-F. & Li, B.-G. (2018) Nutrient-specific compensation for seasonal cold stress in a free-ranging temperate colobine monkey. *Functional Ecology,* **32,** 2170-2180.

Kohl, K.D., Weiss, R.B., Cox, J., Dale, C. & Dearing, M.D. (2014) Gut microbes of mammalian herbivores facilitate intake of plant toxins. *Ecology Letters,* **17,** 1238-1246.

Kurnath, P. & Dearing, M.D. (2013) Warmer ambient temperatures depress liver function in a mammalian herbivore. *Biology Letters,* **9,** 20130562.

Kurnath, P., Merz, N.D. & Dearing, M.D. (2016) Ambient temperature influences tolerance to plant secondary compounds in a mammalian herbivore. *Proceedings of the Royal Society B,* **283,** 20152387.

Marsh, K.J., Moore, B.D., Wallis, I.R. & Foley, W.J. (2014a) Continuous monitoring of feeding by koalas highlights diurnal differences in tree preferences. *Wildlife Research,* **40,** 639-646.

Marsh, K.J., Moore, B.D., Wallis, I.R. & Foley, W.J. (2014b) Feeding rates of a mammalian browser confirm the predictions of a 'foodscape' model of its habitat. *Oecologia,* **174,** 873-882.

Marsh, K.J., Wallis, I.R. & Foley, W.J. (2007) Behavioural contributions to the regulated intake of plant secondary metabolites in koalas. *Oecologia,* **154,** 283-290.

McArthur, C., Banks, P.B., Boonstra, R. & Forbey, J.S. (2014) The dilemma of foraging herbivores: dealing with food and fear. *Oecologia,* **176,** 677-689.

Moore, B.D., Foley, W.J., Wallis, I.R., Cowling, A. & Handasyde, K.A. (2005) *Eucalyptus* foliar chemistry explains selective feeding by koalas. *Biology Letters,* **1,** 64-67.

Moore, B.D., Lawler, I.R., Wallis, I.R., Beale, C.M. & Foley, W.J. (2010) Palatability mapping: a koala's eye view of spatial variation in habitat quality. *Ecology,* **91,** 3165-3176.

Norton, T.W. & Neave, H.M. (1990) Koala habitat: a conceptual functional model. *Koalas – Research for Management* (ed. G. Gordon), pp. 93–101. World Koala Research Inc., Brisbane

Ominski, K.H., Kennedy, A.D., Wittenberg, K.M. & Nia, S.A.M. (2002) Physiological and production responses to feeding schedule in lactating dairy cows exposed to short-term, moderate heat stress. *Journal of Dairy Science,* **85,** 730-737.

Provenza, F.D. & Villalba, J.J. (2006) Foraging in domestic herbivores: linking the internal and external milieux. *Feeding in Domestic Vertebrates: From Structure to Behavior* (ed. V.L. Bels), pp. 210 - 240. CABI Publishing, Oxfordshire, UK.

Provenza, F.D., Villalba, J.J., Dziba, L.E., Atwood, S.B. & Banner, R.E. (2003) Linking herbivore experience, varied diets, and plant biochemical diversity. *Small Ruminant Research,* **49,** 257-274.

Renaudeau, D., Quiniou, N. & Dubois, S. (2002) Effects of high ambient temperature and dietary protein level on feeding behavior of multiparous lactating sows. *Animal Research,* **51,** 227-243.

Rhoads, M.L., Rhoads, R.P., VanBaale, M.J., Collier, R.J., Sanders, S.R., Weber, W.J., Crooker, B.A. & Baumgard, L.H. (2009) Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatotropin. *Journal of Dairy Science,* **92,** 1986-1997.

Rothman, J.M., Chapman, C.A., Struhsaker, T.T., Raubenheimer, D., Twinomugisha, D. & Waterman, P.G. (2015) Long-term declines in nutritional quality of tropical leaves. *Ecology,* **96,** 873-878.

Seabloom, E.W. & Reichman, O.J. (2001) Simulation models of the interactions between herbivore foraging strategies, social behavior, and plant community dynamics. *The American Naturalist,* **157,** 76-96.

Skopec, M.M., Haley, S. & Dearing, M.D. (2007) Differential hepatic gene expression of a dietary specialist (*Neotoma stephens*i) and generalist (*Neotoma albigula*) in response to juniper (*Juniperus monosperma*) ingestion. *Comparative Biochemistry and Physiology Part D:,* **2,** 34-43.

Speakman, J.R. & Król, E. (2010) Maximal heat dissipation capacity and hyperthermia risk: neglected key factors in the ecology of endotherms. *Journal of Animal Ecology,* **79,** 726-746.

Torregrossa, A.-M. & Dearing, M.D. (2009) Nutritional toxicology of mammals: regulated intake of plant secondary compounds. *Functional Ecology,* **23,** 48-56.

Wallis, I.R., Nicolle, D. & Foley, W.J. (2010) Available and not total nitrogen in leaves explains key chemical differences between the eucalypt subgenera. *Forest Ecology and Management,* **260,** 814-821.

White, R.G. (1983) Foraging patterns and their multiplier effects on productivity of northern ungulates. *Oikos,* **40,** 377-384.

Chapter 6

Can plant secondary metabolites act as mitochondrial uncouplers? Implications for heat balance in animals

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Abstract

Plant secondary metabolites (PSMs) are a component of the diets of herbivores. In the marsupial-*Eucalypt* system, herbivores consume leaves with high concentrations of PSMs at every meal. The concentration and mixture of these PSMs is a key determinant of food selection and of how much herbivores are able to eat. Recently, given the changing climate, there has been increased interest in whether ambient temperature may limit the tolerance of herbivores to PSMs. One mechanism for this is through PSMs directly interfering with cellular energy metabolism. If PSMs uncouple mitochondrial oxidative phosphorylation, heat is produced, and at warmer temperatures excess heat is difficult to dissipate. This means that herbivores may be constrained from feeding on PSMs at warmer temperatures. While there have been some *in vitro* studies showing uncoupling by certain PSMs from *Eucalypts* different laboratories have used different protocols including vesicles, to isolated mitochondria, to whole cells. Given the importance of cellular regulation on mitochondria, we test a range of PSMs for mitochondrial uncoupling activity using a single whole cell-based protocol. While we showed that some PSMs tested did interfere with cellular energy metabolism, none acted as strong mitochondrial uncoupling agents. We found the protocol useful to screen for the biological activity of a large number of PSMs at once, however we suggest given the importance of concentration, it cannot be used to rule out biological activity.

Introduction

Browsing mammals ingest a range of plant secondary metabolites (PSMs) in their natural diet of tree and shrub foliage. Many of these compounds act as feeding deterrents against herbivores by exhibiting various biological activities, including anti-nutritive effects, nausea, and direct toxic effects on cells (Dearing, Foley & McLean 2005; Forbey & Foley 2009). There is a growing body of evidence that the biological activity and toxicity of PSMs can be affected by the ambient temperature which an herbivorous mammal experiences. For example, some herbivores (e.g. brushtail possums, ringtail possums, woodrats) exhibit reduced tolerance to PSMs at warm ambient temperatures (Kurnath, Merz & Dearing 2016). This is known as temperature dependent toxicity (Dearing 2013), and may be a response to the endogenous heat produced during PSM metabolism (Beale *et al.* 2018). Dissipation of excess heat is difficult under warm conditions, which limits the rate at which it can safely be produced (Speakman & Król 2010). As well as producing heat via metabolism, another way in which PSMs may increase endogenous heat production is via direct effects on mitochondria. Given the current climate projections for warmer average temperatures, and more frequent heat waves of higher severity, as well as predicted increases in PSM concentrations in *Eucalyptus* trees, understanding the mechanisms by which diet and thermoregulation may interact is important for conservation efforts.

Mitochondria are commonly referred to as the "power houses of the cell" as they are organelles within all cells responsible, among other things, for the production of ATP via cellular respiration a by-product of which is body heat. The proteins responsible for ATP formation include the electron transport chain (Complex I – IV) and ATP synthase (Complex V). They are situated on the inner mitochondrial membrane. ATP synthase relies on the existence of an electrochemical gradient between the inter-membrane space and the mitochondrial matrix, as the flow of protons through ATP synthase allows the energetically unfavorable reaction to occur. The electron transport chain generates this electrochemical gradient through a series of redox reactions that consume oxygen and also generate heat. Uncoupling refers to a mismatch between oxygen consumption by the electron transport chain and ATP production by ATP synthase. It is induced by dissipation of the electrochemical gradient, usually by passage of protons across the

inner mitochondrial membrane. The reactions of the electron transport chain are then upregulated to maintain the electrochemical gradient needed to drive ATP synthase, and consequently more oxygen is consumed and more heat is produced per unit of ATP generated. The body will use uncoupling proteins that allow dissipation of the gradient to upregulate heat production or waste excess energy as heat (Busiello, Savarese & Lombardi 2015). However xenobiotic compounds can also induce uncoupling, either by encouraging the production of endogenous uncoupling proteins (Kida *et al.* 2016), or by acting directly as protonophores that passively transport protons across the inner mitochondrial membrane (Childress *et al.* 2018). Indeed, macrocarpals (terpene-phloroglucinol adducts) from the leaves of *Eucalyptus viminalis* demonstrated protonophoric activity in artificial lipid bilayers and uncoupled isolated mitochondria *in vitro* in rat liver isolates (Spiridonov *et al.* 2003). This had led to the suggestion that other PSMs found in *Eucalyptus* leaves have chemical properties that are common to known protonophores.

Although mitochondrial uncoupling is a well-known phenomenon in neonate mammals responding to the cold, metabolism and obesity research, and in drug development research, it has not previously been considered as a potential mechanism for driving temperature-dependent changes in the feeding behaviour of herbivores. Furthermore, there is some inconsistency in how different PSMs are tested for uncoupling activity, with many possible methodological options in terms of laboratory equipment, whole cells versus isolated mitochondria, which cell type to test, and at what concentration *in vitro* when the *in vivo* range at the cellular level is unknown. The aim of this study was therefore to use the same method to test whether a range of PSMs known to limit food intake by marsupial folivores may act as mitochondrial uncouplers, and therefore increase endogenous heat production. We used whole cells as cellular regulation of mitochondrial function is lost when using isolated mitochondria (Hill *et al.* 2012). To test this hypothesis, we use a XF24-3 Extracellular Flux Analyser (Seahorse Bioscience, MA, USA) to perform titrations of PSMs and determine if they increase the rate of oxygen consumption (OCR) in liver cell culture across this concentration range. Following this, we performed mitochondrial stress tests on cells treated with the PSMs that increased OCR and calculate several bioenergetic measures of cell function. We intended this experiment as a preliminary standardized screening for mitochondrial uncoupling

potential by PSMs. From here, a wider range of PSMs, of concentrations, of cell types, and of techniques could be attempted. We anticipate that further *in vitro* and *in vivo* testing would be needed to further explore the possibility that dietary PSMs could upregulate heat production through mitochondrial uncoupling.

Methods

Cell culture

An immortalized human liver cell line (HEPG2) was purchased from the American Type Culture Collection (ATCC, Virginia, USA). The HEPG2 cell line was derived from a biopsy of a hepatocellular carcinoma from a 15 year old Caucasian male. HEPG2 cells were grown and maintained in growth medium (GM) containing high glucose Dulbecco's modified eagle medium (DMEM; Gibco), foetal bovine serum (10%; Bovogen Biologicals) and antimycotic/antibiotic solution (1%; Sigma Aldrich). Cells were seeded at a confluency of ~10% in a 75cm^2 flask and passaged every 3 days.

Plant secondary metabolites

The PSMs used are displayed in (Table 1). They were selected because their activity (with the exception of curcumin) and had been tested in vivo in feeding experiments with common brushtail possums (Table 1). In addition, the known uncoupling agent DNP was included to provide a positive control

A titration of all compounds was performed across 0.1 - 3μM concentrations prior to the mitochondrial stress test to ensure that the maximal response (whether inhibitory or stimulatory) was achieved (see supplementary material). Compounds were selected for further testing if they increased the OCR above basal OCR and, the concentration selected was that which induced the maximum OCR.

Table 1: *List of compounds used in the experiments together with data on the voluntary intake of the compounds by common brushtail possums (2.0-2.5 kg), the predicted effect of increased ambient temperature on their intake and known mitochondrial effects. *Included as example of PSM already thought to uncouple. Not known from Eucalyptus leaves. **Never reduced intake.*

PSM	Concentration in stress test	Type of PSM	Approx intake (g/day)	Temperature-intake effects	Known mitochondrial effects	References
Benzoic acid	Excluded as did not increase OCR	Aromatic carboxylic acid	0.19	unknown	Weak adenine nucleotide translocase (ANT) dependent uncoupler *in vitro* in isolated mitochondria.	(Au *et al.* 2013) (Lou *et al.* 2007)
Chrysin	Excluded as did not increase OCR	Flavone	1.50	unknown	Weakly in vesicles	(Marsh *et al.* 2015) (van Dijk, Driessen & Recourt 2000)
Curcumin	1μM	Diarylheptanoid	NA*	↓ intake of phenolics with ↑ temp in woodrat-Juniper system	Uncoupler *in vitro* in isolated mitochondria	(Dearing 2013) (Lim, Lim & Wong 2009)
Flavanone	1μM	Unsubstituted B-ring flavanone	0.51	↓ intake after 1wk at ↑ temp	Some weakly in vesicles	(Marsh *et al.* 2015) (van Dijk, Driessen & Recourt 2000)
Gallic acid	0.5μM	Polyphenolic	2.53	↓ intake of phenolics with ↑ temp in woodrat-Juniper system		(Wiggins *et al.* 2003) (Dearing 2013)
Jensenone	0.1μM	Formylated phloroglucinol	0.13	Non-significant ↓ with ↑ temp		(Stapley *et al.* 2000)

		compound (FPC)				
Pinocembrin	Excluded as did not increase OCR	Unsubstituted B-ring flavanone	0.24	Unknown	Weakly in vesicles	(Marsh *et al.* 2015) (van Dijk, Driessen & Recourt 2000)
Rutin	0.1μM	Flavonol glycoside	13.35**	Unknown		(Marsh *et al.* 2006)
Sideroxylonal	1.5μM	Formylated phloroglucinol compound (FPC)	0.45	↓ intake after 1wk at ↑ temp		(Marsh *et al.* 2003)

Mitochondrial stress test

A mitochondrial stress test, which measures mitochondrial function (oxygen consumption rate; OCR) and extracellular acidification rate (indicative of, anaerobic glycolytic metabolism; ECAR) was performed on an XF24-3 Extracellular Flux Analyser (Seahorse Bioscience, MA, USA). The XF24 Flux Analyser measures real-time changes in dissolved oxygen (O_2) and pH over various respiratory states.

Seahorse XF24 cell culture V7 microplates were seeded with 20,000 cells in 100 μL of GM per well and incubated for 3 hours in a 37°C incubator to allow the cells to adhere to the bottom of the well. Once the cells had adhered, 150 μL of GM was added to each well and the plate was returned to the incubator for overnight incubation. Following the overnight incubation, 200 μL of GM was removed from each well and the cells were washed with 1000 μL of 37°C pre-warmed Assay Media (AM; DMEM XF assay media (Seahorse Bioscience), 25 mM glucose and 1 mM sodium pyruvate; pH 7.4; AM). The 1000 μL of AM was removed from each well and replaced with 625 μL of AM to give a final volume of 675 μL per well. 675 μL of AM was added to the background control wells and the microplate was returned to an incubator for 1 hour for pH and temperature equilibration.

Following this, 50 µL of either AM (for untreated wells) or the test PSM dissolved in DMSO was added to injection port A. The final assay concentration of each test compound varied according to the results of the titration. 55 µL of oligomycin was added to injection port B (to give a final assay concentration of 3 µM), 60 µL of FCCP was added to injection port C (to give a final assay concentration of 0.5 µM) and 65 µL of antimycin A and rotenone was added to injection port D (to give a final assay concentration of 1.5 µM and 3 µM, respectively). The loaded Sensor Cartridge was inserted into the XF24 Analyser for calibration and once the calibration was completed, the loaded microplate was inserted and the mitochondrial stress test was commenced. This involved measuring OCR during five different respiration states. In all states, a measurement cycle consisted of a 3 min mix, a 2 min wait and a 3 min data collection period. Three measurement cycles were undertaken during each respiration state.

The first respiration state (basal respiration) took place prior to injection of the PSM. Glucose-driven respiration (basal/State 2) indicates utilisation of endogenous adenosine diphosphate at rest by the electron transport chain (ETC) and includes any OCR driven by the leak of protons (H^+). We then injected the test compounds, to measure the second respiration state (compound-stimulated respiration). A third respiration state was measured by injection of oligomycin A to inhibit the activity of ATP synthase (complex V) of the ETC and induce State 4 respiration. The fourth (maximal, state 3 uncoupled respiration) was measured following addition of FCCP (carbonyl cyanide p-trifluoromethoxy-phenylhydrazone), the most potent known uncoupler of oxidative phospohorylation. FCCP induces maximal respiration by increasing the permeability of the inner mitochondrial membrane to H^+ allowing it to leave the intermembrane space causing the collapse of mitochondrial membrane potential ($\Delta\Psi$). This stimulates the ETC to drive electron transport at its maximum. Finally, inhibited respiration is measured following the addition of rotenone and antimycin A (acting on complex I and III, respectively). These drugs inhibit electron flow along the ETC which completely prevents ETC function. This enables the measurement of non-mitochondrial OCR as a background control. Together, these measurements can be used to calculate several cellular bioenergetic measures.

Figure 1. *Schematic of the mitochondrial stress test showing the sequence at which PSMs, oligomycin, FCCP, and Rotenone and antimycin A are added to the plates. Intervals used in calculation of bioenergetic measures are labelled on the x-axis. Maximal OCR (maximum value in Int3), pre-experiment OCR (maximum value in Int0), initial experimental OCR (Int 1), ATPase independent OCR (minimum value Int2) and ETC-independent or non-mitochondrial OCR (Int 4), are indicated by dashed horizontal lines.*

Statistical analysis

Following the methods described by Yépez et al (2018) well based and point based outliers were removed from the data set and OCR was log transformed. Ratio based bioenergetic metrics were then calculated from the mitochondrial stress test results as per Table 2 (Yépez *et al.* 2018). Two additional metrics, PSM-induced respiration and coupling efficiency, were also calculated to investigate the effect of the PSM on the cells. Each bioenergetics measure was compared to the control treatment by linear mixed models with PSM treatment as the independent variable and well number as the random variable (R studio; Packages: lme4, lmerTest).

Table 2: Abbreviations and descriptions of bioenergetic measured calculated.

Abbreviation	Definition	Metric
E/I	Proportion of OCR in the ETC with respect to the initial OCR. Analogous to basal respiration	$\frac{OCR0 - OCR4}{OCR0}$
A/I	Proportion of OCR driven from ATP-ase proton pumping with respect to initial OCR. Analogous to ATP-linked respiration.	$\frac{OCR0 - OCR2}{OCR0}$
E/Ai	Proportion of OCR in the ETC, but not driven from ATPase proton pumping, with respect to all non-ATPase OCR. Analogous to proton leak.	$\frac{OCR2 - OCR4}{OCR2}$
M/I	Ratio between maximal OCR and initial OCR. Analogous to the spare respiratory capacity.	$\frac{OCR3}{OCR0}$
M/Ei	Ratio between maximal OCR and non-ETC driven OCR. Analogous to maximal respiration	$\frac{OCR3}{OCR4}$
P/I	PSM stimulated respiration as a proportion of initial respiration	$\frac{OCR1 - OCR4}{OCR0}$
P/O	Coupling efficiency	$\frac{(OCR0 - OCR2)}{(OCR2 - OCR4)}$
NM	Non-mitochondrial respiration.	*OCR5*

Results

Mitochondrial stress test

The cells treated with PSMs all responded to the mitochondrial stress test. However, the starting OCR values were different for different treatment groups, therefore the graphs of mitochondrial stress test results are presented for inspection (Figure 2), while statistical analysis was only performed on the bioenergetics metrics.

Bioenergetic measures

There was no difference between treatment and control for any of E/Ai, M/I, M/Ei, P/O, or P/I (Figure 2A, C, D, E, and G). Rutin ($t = -2.693$, $P = 0.01$) and sideroxylonal ($t = -2.276$, $P = 0.028$) treated cells both had lower E/I compared to control cells (Figure 2A). DNP ($t = -9.159$, $P < 0.001$), flavanone (-3.223, $P = 0.002$), and jensenone ($t = -3.580$, $P = 0.001$) treated cells all show significantly lower A/I as compared to the control cells (Figure 2B). E/Ai ($t = -2.346$, $P = 0.024$) and M/Ei ($t = -2.033$, $P = 0.0491$) were both lower compared to control cells in cells treated with rutin (Figure 2C). While all treatments, excepting curcumin, differed from the control cells in non-mitochondrial OCR (Figure 2F). DNP ($t = -2.916$, $P = 0.006$), gallic acid ($t = -2.126$, $P = 0.040$), and jensenone ($t = -2.04$, $P = 0.048$) treated cells have lower NM compared to the control, and rutin ($t = 5.768$, $P <0.001$) and sideroxylonal treated cells ($t = 4.014$, $P< 0.001$) had higher NM compared to the control. Flavanone treated cells trended to lower NM compared to the control ($t= -1.862$, $P = 0.07$).

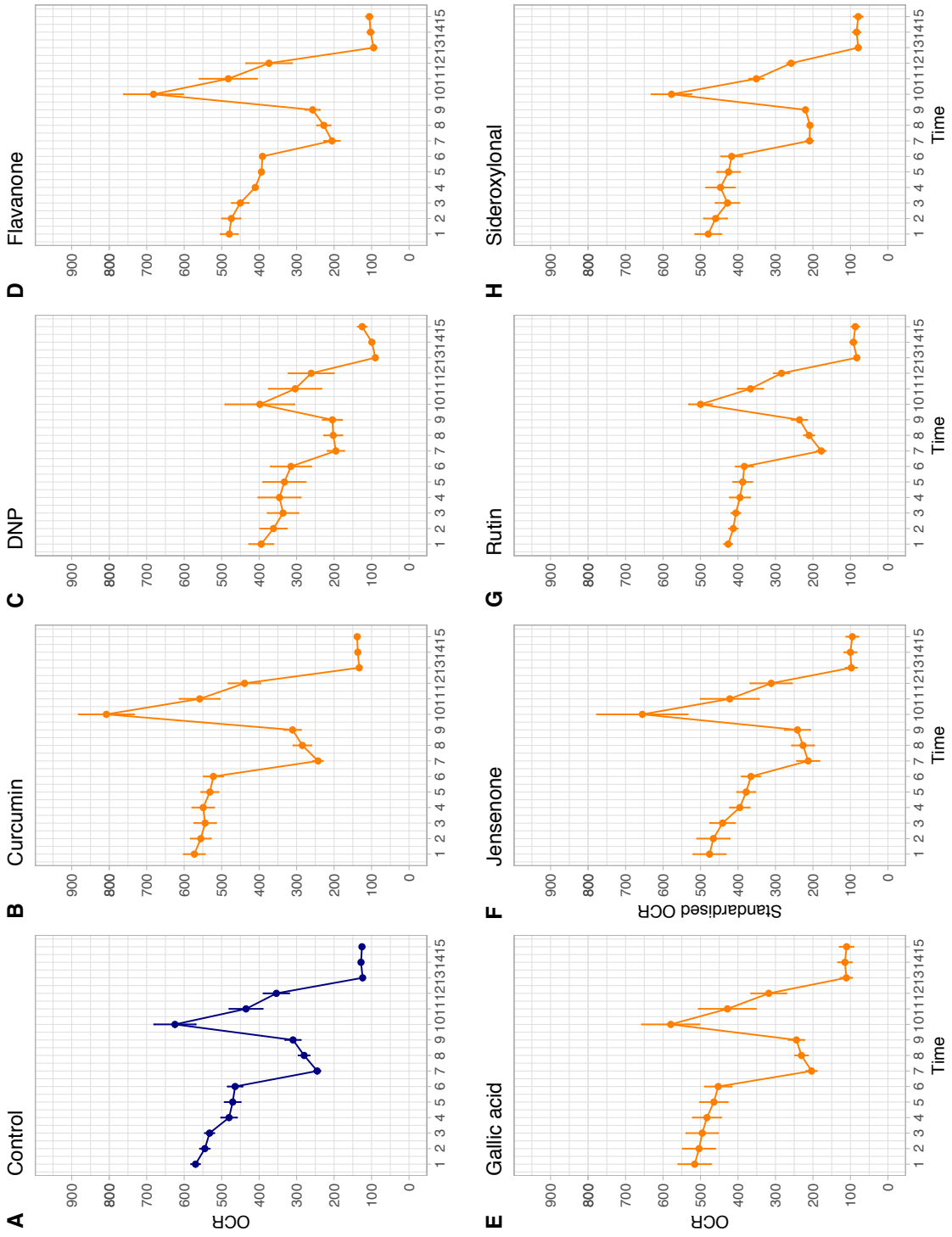

Figure 2. *Mitochondrial stress test results for each PSM compared to the control. For ease of visualization, the stress test results are standardized such that the difference between the control cells and the cells treated with curcumin (A), DNP (B), flavanone (C), gallic acid (D), jensenone (E), rutin (F), and sideroxylonal (G), at time point 1 (before treatment with PSMs) was subtracted at each time point.*

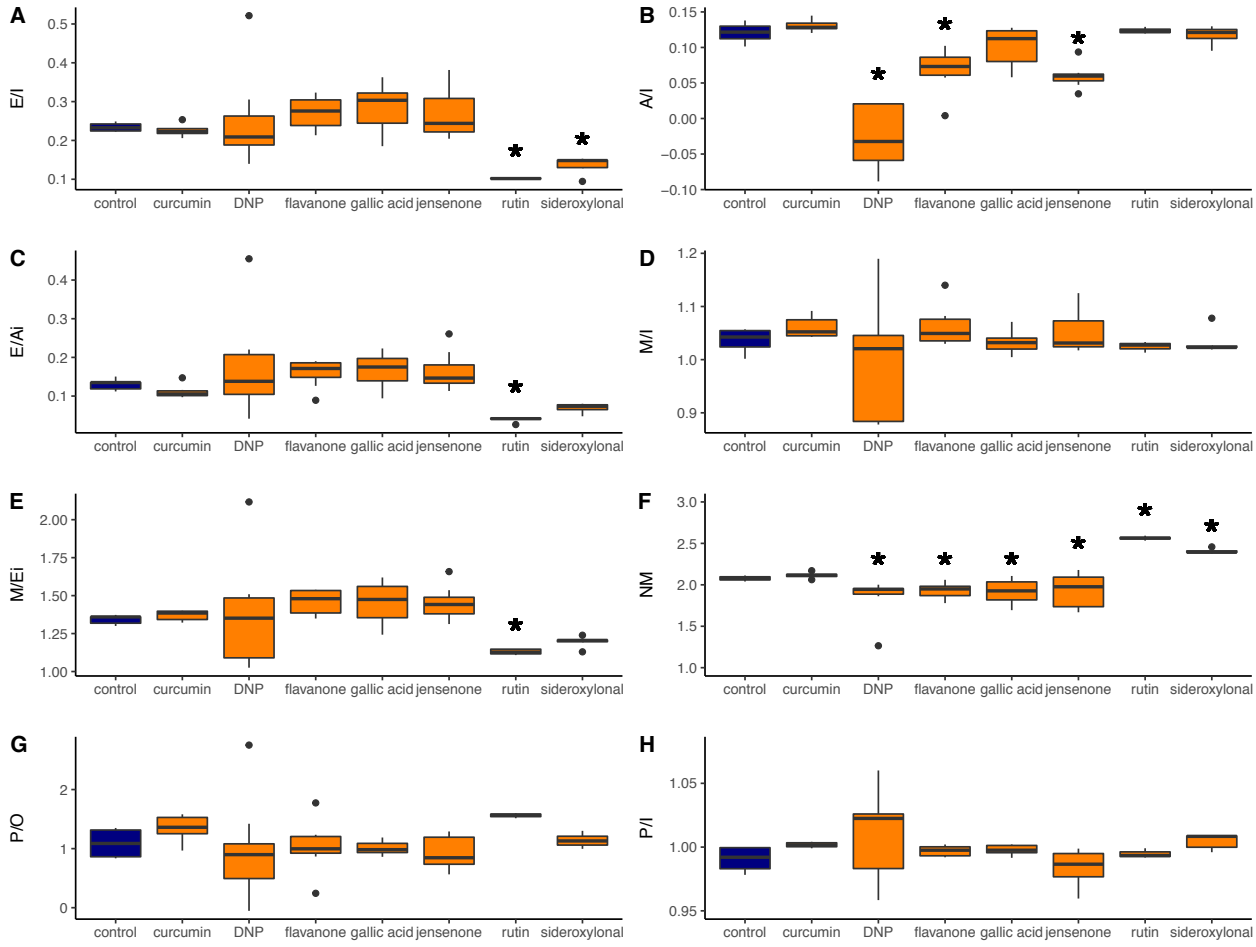

Figure 3. *Bioenergetic measures of cells treated with PSMs (orange) as compared to the control (dark blue). Where the treatment is significantly different from the control is indicated by a star. Bioenergetic measures are calculated based as in Table 1. Panel A represents the proportion of OCR in the ETC with respect to the initial OCR. Panel B represents proportion of OCR driven from ATP-ase proton pumping with respect to initial OCR. Panel C represents proportion of OCR in the ETC, but not driven from ATPase proton pumping, with respect to all non-ATPase OCR. Panel D represents the ratio between maximal OCR and initial OCR. Panel E represents the ratio between maximal OCR and non-ETC driven OCR. Panel F represents non-mitochondrial oxygen consumption, and is the raw OCR for Int5. Panel G represents the coupling efficiency as a proportion of ATP-ase independent OCR. Panel H represents PSM stimulated respiration as a proportion of initial respiration.*

Discussion

The PSMs tested from *Eucalyptus* leaves are not strong mitochondrial uncoupling agents in liver cells at the concentrations we tested, or at the most demonstrate very weak uncoupling activity. The OCR in terms of P/I and P/O was not increased by the addition of any of the PSMs.

Relative to initial OCR, ATP-linked respiration (A/I) is lower when cells were treated with flavanone, jensenone and DNP. This ATP-linked respiration gives an indication of the OCR driven in response to flow of protons through ATP synthase. A decrease in ATP-linked OCR can indicate several things. Firstly, a low ATP demand would reduce the flow of protons through ATP synthase leading to less demand on the ETC to maintain the electrochemical gradient. Secondly a lack of substrate availability to the ETC would lower the rate at which it can function and hence consume oxygen. There is no reason why either of these two scenarios should be occurring in this experiment. The third option is damage to or direct inhibition of oxidative phosphorylation, which would impede the flow of electrons and result in a lower OCR. In the presence of jensenone or flavanone, the maximal respiration following stimulation by FCCP (phase 4) was no different to other treatments, indicating that the electron transport chain had not been impaired by either of these PSMs. Therefore, the reduction in A/I when jensenone or flavanone were present may reflect a partial inhibition of ATP-synthase, similar (although less complete) to that induced by oligomycin. Jensenone possesses two formyl groups which may form Schiff bases with amine groups associated with enzymes. Notably, sideroxylonal, which is a dimer of jensenone, also possesses formyl groups although we did not see any change in A/I in the sideroxylonal treated cells. Previous studies of polyphenolic PSMs have indicated the capacity to bind various ATP-synthase subunits and inhibit its function (Zheng & Ramirez 2000; Hong & Pedersen 2008; Chinnam *et al.* 2010; Gorlach, Fichna & Lewandowska 2015). It is possible, therefore, that jensenone and flavanone act similarly to oligomycin and inhibit ATP-synthase, and that this warrants further investigation.

An alternative view is that jensenone and flavanone act as “couplers” of oxidative phosphorylation by reducing demand on the electron transport chain to maintain the

electrochemical gradient. This could be through donating protons into the intermembrane space, but they have a pKa similar to the other PSMs tested (approximately 3.08) so donation of protons is not more or less likely for any of these PSMs. Or by reducing intrinsic proton leak associated OCR. Basal proton leak (E/Ai) is incompletely understood, but it may exist as a mechanism for countering damage induced by free radical production, and a large part is attributed to the abundance of adenine nucleotide translocase (ANT) (Brand *et al.* 2005). Interactions with ANT have been reported in several other PSMs (E.g. quercin, apigenin) (Gorlach, Fichna & Lewandowska 2015). If jensenone or flavanone reduce basal proton leak we might expect to see a decrease in leak-linked OCR, which was not apparent in this experiment (Figure 3C), although quantification of proton leakiness requires measurement of respiration at a defined Δp.

Non-mitochondrial OCR (NM) was increased in the rutin and sideroxylonal treated cells relative to the control (Figure 3F). Non-mitochondrial OCR reflects enzymatic reactions occurring outside of the mitochondria. These include enzymatic and oxidative processes such as those used for detoxification. Rutin treated cells, but not sideroxylonal treated cells, also have lower P/I (Figure 3H), or OCR stimulated by PSM addition, as compared to the control. Inhibition of OCR by the addition of a PSM could relate to inhibition of ATP-synthase, inhibition of the ETC, reduced ATP demand, or reduced demand on the ETC through proton donation to the intermembrane space. There was no difference in A/I or E/Ai compared to the control for rutin treated cells meaning ATP-synthase and the ETC were not inhibited. Interestingly rutin has been reported in other studies to enhance the action of cytochrome C oxidase, which transports electron between complex III and complex IV, leading to enhanced mitochondrial respiration and cellular energy levels. In addition, rutin consumption by rats increased mitochondrial size and number in skeletal muscle cells as well as adenosine monophosphate-activated protein kinase (AMPK) activity (Seo *et al.* 2015). AMPK in the liver inhibits gluconeogenesis but also increases cytochrome C levels. It could be that the addition of rutin, increased the efficiency of the ETC by enhancing the activity of cytochrome C. Without an increased demand for ATP, increased efficiency along the ETC may be reflected by a lower OCR. Rutin may also act as an alternate energy source as its structure contains the flavanol quercetin and the disaccharide sugar rutinose. If rutin is indeed being used as an

alternative energy source, then we can longer assume the presence of rutin across the entire test at the same concentration as it would be consumed by the cells. However, given the higher NM compared to the control cells, it appears to be present at some unknown concentration at least to the end of the mitochondrial stress test. In experiments with several compounds including DNP, flavanone, gallic acid, and jensenone, NM was decreased relative to the control. Since NM reflects OCR occurring outside of the mitochondria either in the cytosol or on the plasma membrane, a reduction in NM indicates lower overall metabolism in the cell although the exact processes which contribute to NM are not well described.

DNP was included in the assay as a known uncoupler, albeit one that is 500x less potent than FCCP (Terada, 1990), and the results obtained from DNP highlight some of the potential difficulties of exploring the effects of PSMs on cellular metabolism. DNP did not stimulate respiration to levels comparable with the maximal respiration induced by FCCP, however it did reduce A/I and NM relative to untreated controls. At this concentration, there was no significant increase in E/Ai, and hence the P/O of DNP was not different to that of the controls. Of note however, is the high degree of variation within the DNP treatment group, perhaps the results obtained are due to the relatively low concentration of DNP used. For example, in another study the median DNP concentrations required for maximal OCR was between 150 and 350 μM, albeit for a different cell line (Ruas *et al.* 2016). In this experiment, we tested all PSMs within the concentration range recommended for the equipment. While our results did not show strong uncoupling effects within this range, we cannot rule out that these PSMs may cause uncoupling at other concentrations. Our initial concentration titrations clearly show that this type of assay is sensitive to the PSM concentration experienced at a cellular level. In the wild, herbivores eat plants with a variety of PSM concentrations (Marsh *et al.* 2014) and PSMs are broken down to various degrees in the gut (Iason & Murray 1996), are then absorbed in variable amounts (Sorensen & Dearing 2003; Sorensen, Turnbull & Dearing 2004), and then would reach tissues in various concentrations. Extrapolating from the PSM concentration in a plant to the PSM concentration in an animal cell is a formidable task, and we cannot conclusively say how the concentrations used in this experiment would compare to those following ingestion by a wild animal. One difficulty in conducting *in vitro* assays, is knowing exactly what

concentrations to choose, when there is a wealth of unknowns regarding PSM pharmacokinetics, pharmacodynamics, and bioavailability. What we can say, is that there is evidence that PSMs from *Eucalyptus* can directly affect cellular energy metabolism in different ways. They may inhibit ATP-synthase or the ETC, increase or decrease non-mitochondrial oxygen consumption, or they may interact with things that change cellular respiration pathways such as cytochrome C. Therefore, the implications for herbivores consuming PSMs warrants further investigation, and this type of assay may serve as a starting point for other experiments.

In the future, researchers may wish to test a wider range of PSMs and at more varied concentrations to try and encompass what is possible in the wild. One option may be to determine the area under a plasma concentration time curve to gain some sense of bioavailability. Especially given the prospect of first pass metabolism or low bioavailability of orally administered compounds. Furthermore, while we used a hepatic cell line from rats for experimental ease, it would be interesting to repeat the experiment in a possum or other marsupial folivore cell line, given that these are the animals exposed to *Eucalypt* PSMs in the wild. We chose hepatic tissue because compounds absorbed form the gut would be delivered to the liver for metabolism (Dearing, Foley & McLean 2005), and the liver contributes significantly to overall heat production in an animal (Berry *et al.* 1985; Wang *et al.* 2010). However, other tissues may also be exposed to PSMs. For example, the gut wall as a site of first pass metabolism for some PSMs (Dearing, Foley & McLean 2005), and skeletal muscle is a highly thermogenic tissue (Arruda *et al.* 2008; Little & Seebacher 2014; Rowland, Bal & Periasamy 2015). Testing the effects of these PSMs (and others) on possum gut, liver and skeletal muscle cells in a wider variety of concentrations would make this experiment more robust.

Several other approaches have been used to test for uncoupling activity. For example, in the past researchers have opted for using isolated mitochondria, however the current opinion appears to be that the removal of cellular regulation on mitochondrial function that isolating mitochondria necessitates will generate a loss of important information. Further in that direction still, is the use of isolated or artificial lipid bilayers to test for protonophoric activity, which was the approach used by Sprinidov et al (2003), to investigate potential uncoupling activity by terpenoid phenoaldehydes from

E. viminalis. While this can tell us whether the PSM has the potential to act as a protonophor, it is difficult to predict how the PSM will act *in vivo* once further cellular regulation is added. Another future direction is the possibility that while PSMs may not cause acute uncoupling, they may, over time, induce uncoupling proteins which act as channels through which proton flow, bypassing ATP-synthase (Beale *et al.* 2018). Researchers may seek to incubate cells with PSMs to test this hypothesis.

Should a compound found in the natural diet of folivores, show evidence of being a mitochondrial uncoupler *in vitro*, this would warrant future investigation *in vivo*. One possibility is using direct calorimetry to measure heat produced in animals fed diets with no PSMs, with a known uncoupler, and with select PSMs. Most studies to date investigating how mitochondrial uncoupling effects wild animals have focused on life history measures such as growth rates and reproductive success, rather that acute measures of heat production.

The various ways that PSMs may interfere with mitochondrial or cellular function are of course of potential interest, but importantly keeping an eye on the focus of this study, any increase in thermogenesis caused by dietary PSMs may be tolerable under today's climate and pose problems to herbivores consuming them under future temperature regimes. Developing more comparable ways to test for uncoupling will help us to answer this question, and better understand responses to warming temperatures in the animals that consume them.

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References

Arruda, A.P., Ketzer, L.A., Nigro, M., Galina, A., Carvalho, D.P. & de Meis, L. (2008) Cold tolerance in hypothyroid rabbits: Role of skeletal muscle mitochondria and sarcoplasmic reticulum Ca^{2+} ATPase isoform 1 heat production. *Endocrinology,* **149,** 6262-6271.

Au, J., Marsh, K.J., Wallis, I.R. & Foley, W.J. (2013) Whole-body protein turnover reveals the cost of detoxification of secondary metabolites in a vertebrate browser. *Journal of Comparative Physiology B,* **183,** 993-1003.

Beale, P.K., Marsh, K.J., Foley, W.J. & Moore, B.D. (2018) A hot lunch for herbivores: physiological effects of elevated temperatures on mammalian feeding ecology. *Biol Rev Camb Philos Soc,* **93,** 674-692.

Berry, M.N., Clark, D.G., Grivell, A.R. & Wallace, P.G. (1985) The contribution of hepatic metabolism to diet-induced thermogenesis. *Metabolism Clinical and Experimental,* **34,** 141-147.

Brand, M.D., Pakay, J.L., Ocloo, A., Kokoszka, J., Wallace, D.C., Brookes, P.S. & Cornwall, E.J. (2005) The basal proton conductance of mitochondria depends on adenine nucleotide translocase content. *Biochemical Journal,* **392,** 353-362.

Busiello, R.A., Savarese, S. & Lombardi, A. (2015) Mitochondrial uncoupling proteins and energy metabolism. *Frontiers in physiology,* **6,** 36-36.

Childress, E.S., Alexopoulos, S.J., Hoehn, K.L. & Santos, W.L. (2018) Small Molecule Mitochondrial Uncouplers and Their Therapeutic Potential. *Journal of Medicinal Chemistry,* **61,** 4641-4655.

Chinnam, N., Dadi, P.K., Sabri, S.A., Ahmad, M., Kabir, M.A. & Ahmad, Z. (2010) Dietary bioflavonoids inhibit Escherichia coli ATP synthase in a differential manner. *International Journal of Biological Macromolecules,* **46,** 478-486.

Dearing, M.D. (2013) Temperature-dependent toxicity in mammals with implications for herbivores: a review. *Journal of Comparative Physiology B* **183,** 43-50.

Dearing, M.D., Foley, W.J. & McLean, S. (2005) The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. *Annual Review of Ecology Evolution and Systematics,* **36,** 169-189.

Forbey, J.S. & Foley, W.J. (2009) PharmEcology: A pharmacological approach to understanding plant-herbivore interactions. *Integrative and Comparative Biology,* **49,** 267-273.

Gorlach, S., Fichna, J. & Lewandowska, U. (2015) Polyphenols as mitochondria-targeted anticancer drugs. *Cancer Letters,* **366,** 141-149.

Hill, B.G., Benavides, G.A., Lancaster, J.R., Jr., Ballinger, S., Dell'Italia, L., Jianhua, Z. & Darley-Usmar, V.M. (2012) Integration of cellular bioenergetics with mitochondrial quality control and autophagy. *Biological Chemistry,* **393,** 1485-1512.

Hong, S. & Pedersen, P.L. (2008) ATP synthase and the actions of inhibitors utilized to study its roles in human health, disease, and other scientific areas. *Microbiology and molecular biology reviews : MMBR,* **72,** 590-641.

Iason, G.R. & Murray, A.H. (1996) The energy costs of ingestion of naturally occurring nontannin plant phenolics by sheep. *Physiological Zoology,* **69,** 532-546.

Kida, R., Yoshida, H., Murakami, M., Shirai, M., Hashimoto, O., Kawada, T., Matsui, T. & Funaba, M. (2016) Direct action of capsaicin in brown adipogenesis and activation of brown adipocytes. *Cell Biochemistry and Function,* **34,** 34-41.

Kurnath, P., Merz, N.D. & Dearing, M.D. (2016) Ambient temperature influences tolerance to plant secondary compounds in a mammalian herbivore. *Proceedings of the Royal Society B,* **283,** 20152387.

Lim, H.W., Lim, H.Y. & Wong, K.P. (2009) Uncoupling of oxidative phosphorylation by curcumin: implication of its cellular mechanism of action. *Biochemical and Biophysical Research Communications,* **389,** 187-192.

Little, A.G. & Seebacher, F. (2014) The evolution of endothermy is explained by thyroid hormone-mediated responses to cold in early vertebrates. *Journal of Experimental Biology,* **217,** 1642-1648.

Lou, P.-H., Hansen, B.S., Olsen, P.H., Tullin, S., Murphy, M.P. & Brand, M.D. (2007) Mitochondrial uncouplers with an extraordinary dynamic range. *The Biochemical journal,* **407,** 129-140.

Marsh, K.J., Foley, W.J., Cowling, A. & Wallis, I.R. (2003) Differential susceptibility to Eucalyptus secondary compounds explains feeding by the common ringtail (*Pseudocheirus peregrinus*) and common brushtail possum (*Trichosurus vulpecula*). *Journal of Comparative Physiology B,* **173,** 69-78.

Marsh, K.J., Moore, B.D., Wallis, I.R. & Foley, W.J. (2014) Continuous monitoring of feeding by koalas highlights diurnal differences in tree preferences. *Wildlife Research,* **40,** 639-646.

Marsh, K.J., Wallis, I.R., McLean, S., Sorensen, J.S. & Foley, W.J. (2006) Conflicting demands on detoxification pathways influence how common brushtail possums choose their diets. *Ecology,* **87,** 2103-2112.

Marsh, K.J., Yin, B., Singh, I.P., Saraf, I., Choudhary, A., Au, J., Tucker, D.J. & Foley, W.J. (2015) From Leaf Metabolome to In Vivo Testing: Identifying Antifeedant Compounds for Ecological Studies of Marsupial Diets. *J Chem Ecol,* **41,** 513-519.

Rowland, L.A., Bal, N.C. & Periasamy, M. (2015) The role of skeletal-muscle-based thermogenic mechanisms in vertebrate endothermy. *Biological Reviews of the Cambridge Philosophical Society,* **90,** 1279-1297.

Ruas, J.S., Siqueira-Santos, E.S., Amigo, I., Rodrigues-Silva, E., Kowaltowski, A.J. & Castilho, R.F. (2016) Underestimation of the Maximal Capacity of the Mitochondrial Electron Transport System in Oligomycin-Treated Cells. *PLOS ONE,* **11,** e0150967.

Seo, S., Lee, M.-S., Chang, E., Shin, Y., Oh, S., Kim, I.-H. & Kim, Y. (2015) Rutin Increases Muscle Mitochondrial Biogenesis with AMPK Activation in High-Fat Diet-Induced Obese Rats. *Nutrients,* **7**.

Sorensen, J.S. & Dearing, M.D. (2003) Elimination of plant toxins by herbivorous woodrats: revisiting an explanation for dietary specialization in mammalian herbivores. *Oecologia,* **134,** 88-94.

Sorensen, J.S., Turnbull, C.A. & Dearing, M.D. (2004) A specialist herbivore (Neotoma stephensi) absorbs fewer plant toxins than does a generalist (Neotoma albigula). *Physiological and Biochemical Zoology,* **77,** 139-148.

Speakman, J.R. & Król, E. (2010) Maximal heat dissipation capacity and hyperthermia risk: neglected key factors in the ecology of endotherms. *Journal of Animal Ecology,* **79,** 726-746.

Spiridonov, N.A., Arkhipov, V.V., Foigel, A.G., Shipulina, L.D. & Fomkina, M.G. (2003) Protonophoric and uncoupling activity of royleanones from *Salvia officinalis* and euvimals from *Eucalyptus viminalis*. *Phytotherapy Research,* **17,** 1228-1230.

Stapley, J., Foley, W.J., Cunningham, R. & Eschler, B. (2000) How well can common brushtail possums regulate their intake of *Eucalyptus* toxins? *Journal of Comparative Physiology B,* **170,** 211-218.

van Dijk, C., Driessen, A.J.M. & Recourt, K. (2000) The uncoupling efficiency and affinity of flavonoids for vesicles. *Biochemical Pharmacology,* **60,** 1593-1600.

Wang, Z., Ying, Z., Bosy-Westphal, A., Zhang, J., Schautz, B., Later, W., Heymsfield, S.B. & Müller, M.J. (2010) Specific metabolic rates of major organs and tissues across adulthood: Evaluation by mechanistic model of resting energy expenditure. *American Journal of Clinical Nutrition,* **92,** 1369-1377.

Wiggins, N.L., McArthur, C., McLean, S. & Boyle, R. (2003) Effects of Two Plant Secondary Metabolites, Cineole and Gallic Acid, on Nightly Feeding Patterns of the Common Brushtail Possum. *Journal of Chemical Ecology,* **29,** 1447-1464.

Yépez, V.A., Kremer, L.S., Iuso, A., Gusic, M., Kopajtich, R., Koňaříková, E., Nadel, A., Wachutka, L., Prokisch, H. & Gagneur, J. (2018) OCR-Stats: Robust estimation and statistical testing of mitochondrial respiration activities using Seahorse XF Analyzer. *PLoS ONE,* **13,** e0199938.

Zheng, J. & Ramirez, V.D. (2000) Inhibition of mitochondrial proton F0F1-ATPase/ATP synthase by polyphenolic phytochemicals. *British Journal of Pharmacology,* **130,** 1115-1123.

Appendix

Compound Titration method

A titration of all compounds was performed prior to the mitochondrial stress test to ensure that the maximal response (whether inhibitory or stimulatory) was achieved.

Seahorse XF24 cell culture V7 microplates were seeded with 20,000 cells in 100µL of GM per well and incubated for 3 hours in a 37°C incubator to allow the cells to adhere to the bottom of the well. Once the cells had adhered, 150µL of GM was added to each well and the plate was returned to the incubator for overnight incubation. Following the overnight incubation, 200µL of GM was removed from each well and the cells were washed with 1000µL of 37°C pre-warmed Assay Media (AM; DMEM XF assay media (Seahorse Bioscience), 25mM glucose and 1mM sodium pyruvate; pH 7.4; AM). The 1000µL of AM was removed from each well and replaced with 625µL of AM to give a final volume of 675µL per well. 675µL of AM was added to the background control wells and the microplate was returned to a non-CO_2 incubator for 1 hour for pH and temperature equilibration.

Each compound was titrated at six concentrations in DMSO in the range of 0.1-3µM by serially diluting 2.5mM stock solutions. 75µL of each dilution was added to Port A of a pre-hydrated XF24 Sensor Cartridge (see Figure One for plate layout). For blank wells, 75µL of AM was added to Port A. The loaded Sensor Cartridge was incubated at 37°C for 10 minutes and then inserted into the XF24 Analyser for calibration. Once calibration was completed, the loaded microplate was inserted into the XF24 Analyser and the compound titration was initiated. Each measurement cycle consisted of a 3 min mix, a 2 min wait and a 3 min data collection period. The measurement cycle was repeated

three times for each titration during both the basal respiration phase (no PSM) and during compound-stimulated respiration following injection of the PSM.

For each compound, the data was analysed to determine at which concentration the maximum stimulation or inhibition of oxygen consumption rate (OCR) was achieved. Any compound which failed to stimulate OCR above basal was omitted from further testing.

Table S1. *Serial dilution of compounds for compound titrations.*

Tube #	Injection Concentration (µM)	Working Concentration (µM)	Assay Medium (µL)	Volume from Previous Tube (µL)
1	30	3	988	-
2	15	1.5	500	500
3	10	1	333	667
4	7.5	0.75	250	750
5	5	0.5	333	667
6	1	0.1	800	200

	1	2	3	4	5	6
A	Blank	0.1	1.5	0.1	0.1	1
B	0.1	0.5	1.5	Blank	1	1.5
C	0.5	1	Blank	0.5	1.5	3
D	1	3	3	0.5	3	Blank

Figure S1*. Plate layout for compound titrations. Two compounds were tested at once-compound one on the left of the plate (columns 1-3) and compound two on the right of the plate (columns 4-6).*

Table S2. Protocol utilised for compound titrations.

Command	Time (min)	Port	Stimulator/Inhibitor	Measurement
Calibrate	10			
Equilibrate				
Mix	3			Basal Respiration
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			
Inject		A	Assay Media/Compound	
Mix	3			Compound-stimulated Respiration
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			

Compound titration results

Within the concentration range tested, flavanone, gallic acid, rutin, curcumin, jensenone and sideroxylonal increased OCR above that of the basal level (Figure S2) as a percentage of the Basal OCR. These compounds were selected for the mitochondrial stress test at the concentration which maximally increased OCR as a percentage of basal. This was 1µM flavanone, 0.5µM gallic acid, 0.1µM rutin, 1µM curcumin, 0.1µM jensenone and 1.5µM sideroxylonal, and 0.1µM DNP.

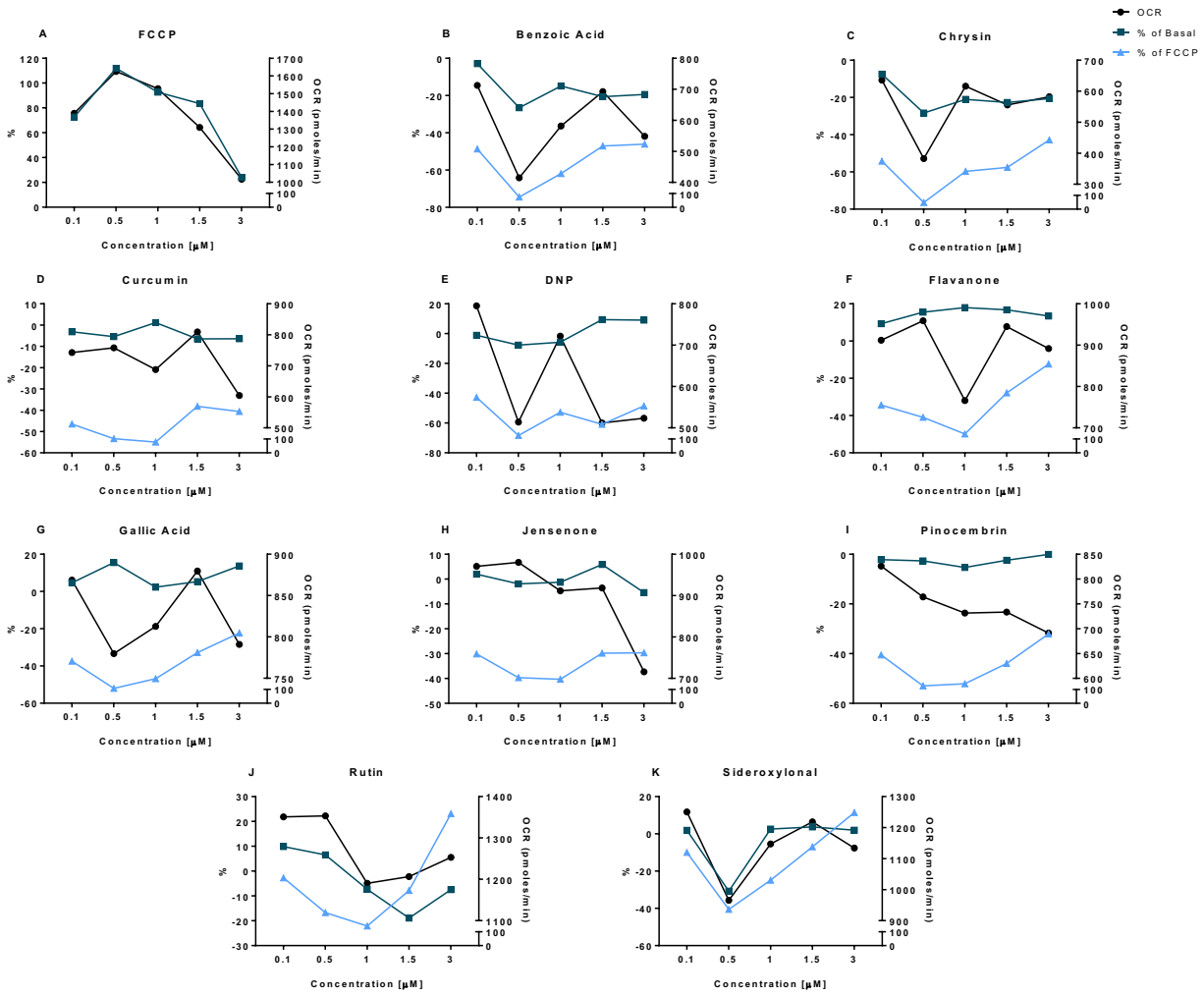

Figure S2. The effect of different concentrations of FCCP and PSMs on OCR as raw values (right axis), and as a percentage of basal respiration (left axis).

Table S3. Protocol utilised for the Mitochondrial Stress Test.

Command	Time (min)	Port	Stimulator/Inhibitor	Measurement
Calibrate	10			
Equilibrate				
Mix	3			Basal Respiration
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			
Inject		A	Assay Media/Uncoupler	
Mix	3			Test-compound-stimulated Respiration
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			
Inject		B	Oligomycin	
Mix	3			Blocked ATP synthase
Wait	2			

Measure	3			
Mix	3			
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			
Inject		C	FCCP	
Mix	3			Maximal Respiration
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			
Inject		D	Antimycin A & Rotenone	
Mix	3			Inhibited Respiration
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			
Mix	3			
Wait	2			
Measure	3			

Synthesis

The nutritional decisions of herbivores are inextricably tied to the thermal environment they experience. This pertains not just to the total energy eaten, but also to the macronutrients from which they choose and to the PSMs that complicate their decision. In this thesis, I have shown that the relationship between thermal physiology and nutritional ecology runs deeper than simply adjusting food intake to meet changes in metabolic rate. Instead, at warm temperatures the intake patterns of herbivores may be guided by limitations rather than requirements, and these limitations can arise in an exposure time dependent way. I will use these concluding remarks not to re-emphasise the results and discussions of each previous chapter, but to link the findings together and to highlight areas of future research.

Ambient temperature influences food intake. While it is in no way surprising that mammals eat more in the cold to match higher thermoregulatory demands, they also eat less in the heat. In the classical model of thermoregulation, mammals experience an increase in metabolic rate above thermoneutrality. Why then would they decrease food intake? Reduced food intake within and above the thermoneutral zone highlights the importance of considering as distinct, behaviours that can be determined by requirements, and behaviours dictated by limitations. Put simply, feeding in the heat brings limitations that must be managed. In the experiments presented in this thesis, possums consistently ate less when housed at warmer temperatures (Chapters 3, 4, 5). This reduction in food intake resulted in weight fluctuations; loss after a week at warm temperatures and gain after a week at cooler temperatures. Interestingly, the changes in food intake, and hence the weight fluctuations, were far smaller in the macronutrient choice experiment in Chapter 4 relative to possums in chapters 2, and 3, even for the same individual possums (Figure 1). This indicates several things; firstly, when animals are *not* given a choice of foods, the only way they can mitigate the effect of heat is by eating less. Secondly, when animals *are* given a choice of foods they can also mitigate the effect of heat by choosing to eat different combinations of foods. This agrees with the findings of others that choice of foods within an environment allow individuals to mix diets that suit their individual needs (Provenza *et al.* 2003), and nutrient balancing and PSM ingestion are linked in animals given a choice (Villalba & Provenza 2005). I questioned at first whether the weight loss experienced by possums in the warm treatments when given no choice was an indication of a limitation on intake or was an

intentional loss of mass to allow for easier heat dissipation. However, the smaller mass loss when possums were given a choice of diets, indicates that animals able to balance their own macro-nutrient intakes are better able to meet their energetic requirements, even when limited by the heat. Again, this reminded me that heat is imposing a limitation on food intake.

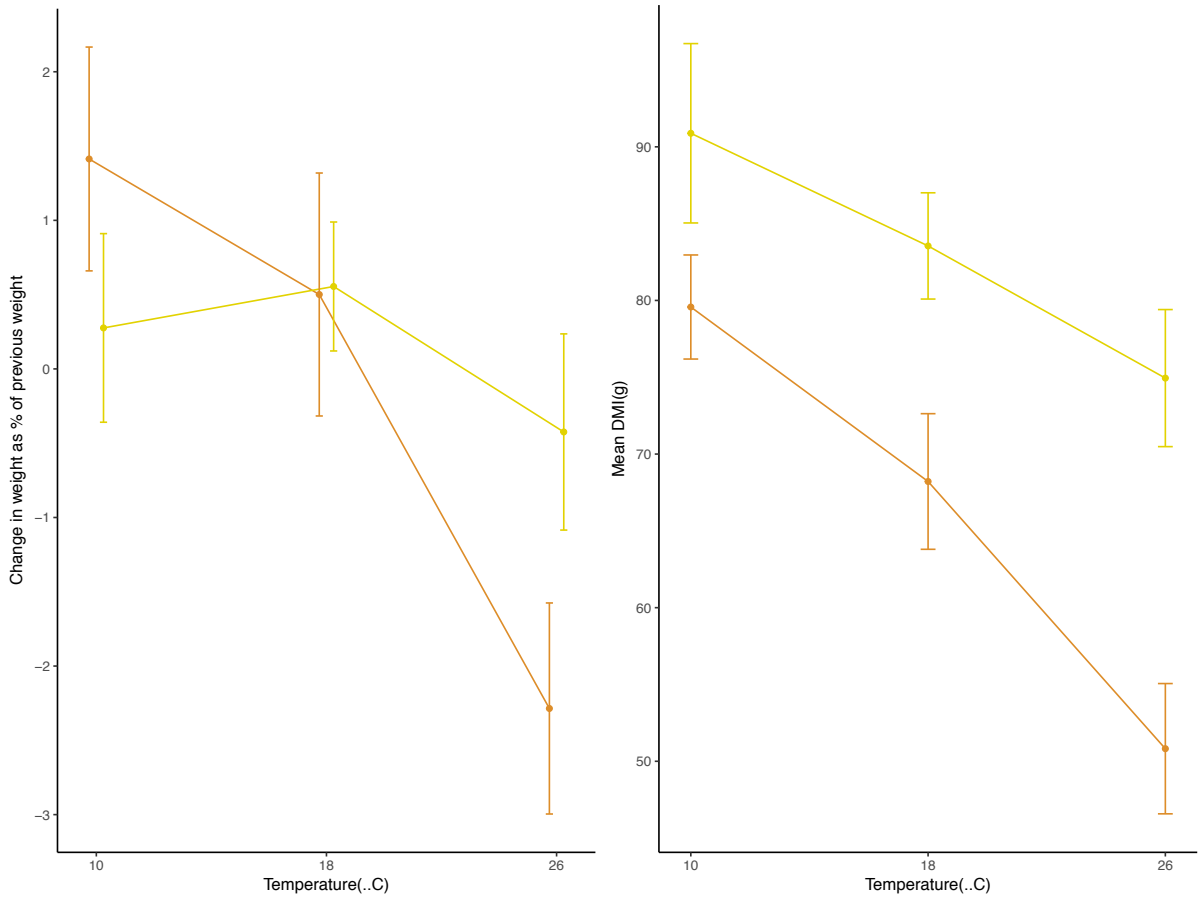

Figure 1: *Mean +/- SE a) changes in the weights of possums (weighed weekly between experimental rounds), and b) daily DMI when possums were kept at three different ambient temperatures for one week, and given no choice (orange) or a choice of diets with different macronutrient compositions (yellow). The data presented for "no choice" are the basal diet intakes measured during the long exposure flavanone study in Chapter 2, and the data for the "choice" are the basal diets fed during the cineole choice experiment in Chapter 4.*

Animals experience ambient temperatures on a continuum from cold to hot, and, while I was interested in the effects of heat, cold temperatures may also influence diet selection. There has been a limited amount of research on how cold or heat may change nutrient selection, however, given the information available from my results

(Chapter 4) and the recent work of others (E.g Guo *et al.* 2018), I suggest intake patterns may follow the hypothetical example presented in Figure 2. I predict that at relatively unchallenging temperatures within the thermoneutral zone, any changes in energy expenditure are met with changes in overall food intake without changing the balance of nutrients. At warm temperatures, the challenge of heat dissipation limitation imposes a reduction in overall food intake and in protein intake to minimise diet induced thermogenesis. At cooler temperatures, increases in thermoregulatory requirements cause increased intake coupled with a change in diet selection towards an increased proportion of readily available non-protein energy intake (Guo *et al.* 2018). This means that, relative to thermoneutral temperatures, both heat and cold challenge cause the protein to non-protein balance in the diet to shift in the same direction.
There is huge opportunity for future experiments in this area. For example, it would be extremely valuable to test a wider range of temperatures and in smaller increments to verify these predictions. Following this, considering how animals in the wild exposed to variable temperatures and other challenges adjust their diet selection, would no doubt prove fruitful.

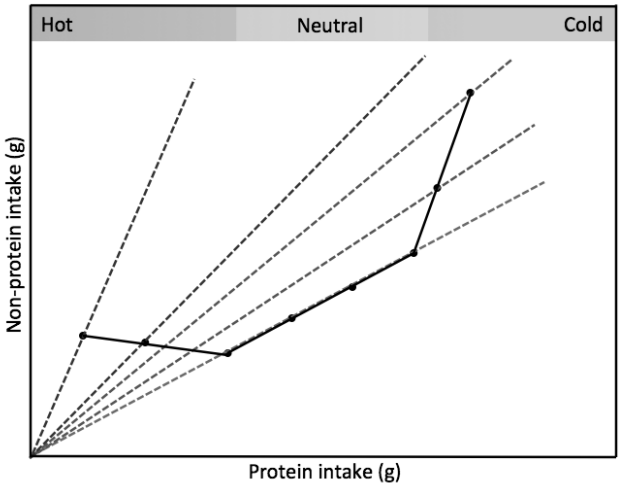

Figure 2: A hypothetical model predicting how animals may change their balance and intake of protein and non-protein energy with ambient temperature (solid black lines). Nutritional rails for a few selected intake points (black dots) are shown as dashed lines. Note that animals may select the same nutritional rail in hot or in cold conditions but consume vastly different amounts.

In chapter 4 I showed that when a PSM was included in the diet, possums chose a different diet composition again; one that was higher in protein, regardless of temperature. While the likely reason for this is an increase in whole-body protein costs of ingesting PSMs, it also demonstrates that one factor may drive a feeding decision one way, and another factor may drive it a different way. These things can be tested in isolation in the lab, but the reality of natural conditions is that there is a constant

tradeoff between opposing forces. So, while I have shown that temperature is a driver of both nutrient selection and PSM tolerance by herbivores, I also reemphasize that the components of the diet cannot be considered in isolation from one another, and that the decisions of the herbivore cannot be viewed as static. Nutrients, PSMs, and temperature can all drive requirements or impose limitations that then culminate in the diet selected by wild herbivores.

I have shown that the intake of potentially toxic diets containing PSMs depends not only on ambient temperature but also on the length of exposure time to those temperatures, while the impact of temperature on intake of PSM-free diets was not changed by exposure time. The experiment in chapter 2 shows that at least one mechanism for the reduction in intake of PSM containing diets following one week of heat exposure is a reduction in hepatic metabolism of xenobiotics. As discussed in chapters 2 and 3, the size of an animal would likely change the effect of exposure time, such that smaller animals are quicker to make physiological adjustments to temperature than are larger animals. The effect of exposure time is interesting, as I typically think of acclimation to conditions as being a series of beneficial physiological changes across the course of exposure making an animal more tolerant to those conditions. At first glance, a reduced tolerance to toxins following longer exposure time, and hence a reduced intake of food containing PSMs, appears detrimental. However, hyperthermia, due to excess heat produced by diet induced thermogenesis, is likely to have more immediate consequences than eating less. The likelihood of hyperthermia can be reduced by eating less food, and by taking in and metabolizing fewer PSMs. Becoming intoxicated is also likely to have more immediate detrimental consequences than eating less. Eating more can wait for times when there is less of a trade off with thermoregulation.

Wild herbivores are exposed to fluctuating rather than constant ambient temperatures. The influence of temperature on nutrition may be different when minimum and maximum temperatures vary within a day. In chapter 5 I showed that on a per-meal basis in koalas, there is an interaction between PSM concentration and the impact of higher ambient temperatures at the time of the meal on per meal food intake, such that if PSM concentration was high, warmer temperatures reduced food intake. It is important to note that in the captive studies of Chapters 3 and 4 only total food intake

per day was measured for each possum. It is possible that, even though there was no difference in PSM intake after a short exposure to different ambient temperatures, possums may have eaten smaller meals but compensated by eating more frequently, leading to no change in food intake within a day. Nevertheless, in Chapter 5, there was no change in the number of meals eaten by koalas on days with different mean, minimum, or maximum temperatures. There was, however, a significant reduction in daily food intake when the mean ambient temperature was warmer, due to changes in meal size rather than changes in meal frequency. This is interesting because it means that the effect of temperature on food intake is at least partly due to immediate feedback to the animals, and points to possibilities for future research at different time scales with a larger and more focused data set.

Of note, is that the temperatures I used in Chapters 2-4 (10 °C, 18 °C and 26 °C) are relatively mild considering the natural range of temperatures that occurs in Canberra (e.g. average daily winter temperature of 1 – 12˚C and average summer daytime temperature of 12 -27˚C), where the possums used in my research were captured. Nevertheless, the temperatures were selected based on the physiological responses of possums to those temperatures. When attempting to characterize the TNZ of possums I did not find an increase in metabolic rate at warmer temperatures like in the classical model, but I did find an increase in body temperature, indicating a limit in thermoregulation. According to my measurements, the warmest temperature to which I exposed possums in the feeding experiments was around the upper critical temperature of the TNZ. The mild nature of these temperatures demonstrates that ambient temperature is likely to affect the feeding choices and PSM tolerance of a wide range of herbivore species residing in many different types of habitats. It also highlights that temperatures used in these types of studies should be of ecological and physiological relevance to the animal in question. So, while I consider the mechanisms discussed in this thesis to drive changes in feeding behavior with temperature to be transferrable to other animals, the specific details in terms of timing and magnitude of temperature differences may vary species to species. In addition, the diets and PSMs used should also be relevant to the specific animal in question.

In Chapter 6 I did not see clear evidence that any of the compounds tested acted as mitochondrial uncouplers. The question of whether PSMs act as mitochondrial

uncouplers comes from an acknowledgement that, during the process of an animal consuming, metabolizing and excreting a PSM, the PSM itself is not an inert substance. Rather, it has biological activity of its own, which is often uncharacterized. Understanding whether a specific PSM is likely to cause an increase in heat production in a herbivore due to mitochondrial uncoupling could be of significant benefit in linking or predicting the effects of elevated ambient temperatures on the feeding behavior of herbivores. Chapter 6 can be considered exploratory, and provides some guidance for future research. For example, first, it would be advantageous to develop possum cell lines from a few different tissues. However, developing cell lines from non-model species is not straightforward, and is likely to take a significant investment of time and resources. Second, although the instrument used to characterize cellular respiration has been widely used in this research area, it was not entirely suitable for cell lines that are suspended. An alternative might be the OROBOROS oxygraph. Third, it would be useful to test a wider range of concentrations, and a wider range of compounds, since mitochondrial uncoupling can be concentration specific, as seen from the DNP results. Fourth, it would be valuable to do a follow up experiment in which cells are incubated with the PSMs to see if they can induce uncoupling proteins. I did see some interesting results that gave rise to sufficient follow up questions to warrant future studies continuing the exploration. For example, is a rise in non-mitochondrial respiration in cells treated with some compounds reflecting detoxification processes? direct calorimetry may be a method for investigating further, to what degree specific PSMs impact heat production at the whole animal level either through detoxification or through uncoupling of mitochondria.

Even small changes in nutrient intake at the individual animal level can have large impacts at the population level. For example, small changes in protein or available protein in the diet can have huge impacts on population demographics and reproductive outcomes in herbivores (White 1983). Likewise, plant secondary metabolites can dictate habitat suitability and herbivore population densities (E.g. Lawler, Foley & Eschler 2000; DeGabriel *et al.* 2009; Youngentob *et al.* 2011; Frye *et al.* 2013). What this means on a broader scale is that the same forest of trees with unchanged chemistry is less edible after a week of warm temperatures, because of changes in animal physiology. It also means that in warm conditions herbivores eat less

overall (Chapters 2-5) and select trees with lower PSM and available N levels (Chapter 5). In warm temperatures, herbivores may be more selective for trees with lower PSM concentrations (Chapter 5), which is only possible if their habitat contains enough low PSM trees. Eating less means fewer nutrients available for other functions (e.g. growth, reproduction) and my data suggests that they do not compensate for this my eating more nutrient rich leaves (Chapter 5). This may result in potential flow on effects in the animal population. Being more selective means an increase in herbivore pressure on individual trees with low PSM concentrations in the environment, leading to flow on effects in the plant population. I have also shown that PSMs can directly interfere with cellular energy metabolism (Chapter 6), although not in the way I hypothesized. Although we don't yet fully understand the relevance of this for wild populations, animals consuming PSMs may be balancing different internal heat production with the external thermal environment, depending on the composition of their diet.

Future directions

Laboratory based

While I explored some mechanisms by which temperature may interact with the diet, I have opened the door to many other angles for future research (Figure 3). Two such areas are blood flow and water balance. Both water and blood link thermoregulation with food intake. Blood distributes oxygen, nutrients, hormones, heat, metabolites, and water around the body. When ambient temperatures are high, a larger pool of blood circulates in the periphery as a heat loss mechanism (Johnson, Minson & Kellogg Jr. 2014), and though perhaps subtle, over time this may change the structure and function of tissues. A smaller centrally circulating blood pool and reduced blood flow to the intestinal wall (Johnson, Minson & Kellogg Jr. 2014), can be the rate-limiting step for the absorption of orally administered drugs (Pang 2003). Reduced intestinal blood flow also means a slower delivery of components absorbed from the gut to the liver (Wilkinson 1975). Second, it has been shown that, over time, the intestinal villi shorten in length in response to extended exposure to warmer ambient temperatures, leaving a smaller absorptive surface area within the gut (Mitchell & Carlisle 1992). Third, slower gut transit time in response to increased ambient temperature may also slow gastric

emptying (Christopherson & Kennedy 1983). There are discrepancies in whether the changes to the gut induced by heat exposure result in increased or decreased digestibility, and changes to absorption per unit of area of the gut can be increased for specific nutrients (e.g. hexoses, amino acids, glucose, Mitchell & Carlisle 1992). But these changes to absorption only ever represent a partial compensation for reduced blood flow, shortened villi, and slowed transit time, so the time in which a PSM persists in the gut would likely be increased at warmer ambient temperatures (Mitchell & Carlisle 1992). It would therefore be interesting to relate temperature to blood flow and investigate whether this could act as a unifying mechanism underpinning all of the mechanisms discussed in chapters 2 through 5 to culminate in an effect of temperature on food and PSM intake by herbivores.

Once absorbed, nutrients and some PSMs from the gut are delivered via the portal vein to the liver for metabolism. Slower absorption from the gut, and slower delivery to the liver, means ingested PSMs could be expected to take longer to reach peak blood concentration and to be cleared from the body (Chillistone & Hardman 2017). Depending on how an ingested PSM is regulated and where it acts in the body, or if there is any storage, a prolonged presence may lead to a more deterrent effect. Nausea signals are a known mechanism by which some PSMs deter feeding (Provenza *et al.* 1994). For example, possums ingesting *Eucalyptus* leaves ate significantly more of the PSM jensenone (a compound of the same class and action as sideroxylonal) when dosed with a selective 5-HT_3 receptor antagonist to prevent nausea (Lawler *et al.* 1998; DeGabriel *et al.* 2010).

Figure 3: *Flow diagram showing how I see the factors explored in this thesis (marigold boxes) are connected with already established knowledge (turquoise boxes) and with ideas explored in isolation in other study systems for which I suggest future research could be directed (orange boxes).*

For PSMs that exert effects from within the gut, a slower absorption may lead to a prolonged deterrence from feeding, leading to lower overall intake by the animal. This would not necessarily increase overall toxicity. For instance, if a PSM deters feeding

from within the gut, but exerts toxic effects following absorption, prolonged persistence within the gut would increase deterrence, but the PSM would have a lower peak blood concentration (figure 4). Therefore, another area for future research is gut transit or intestinal absorption studies coupled with blood flow studies to determine the exact mechanisms by which exposure to ambient temperatures of different durations acts to change the intake of diets containing PSMs.

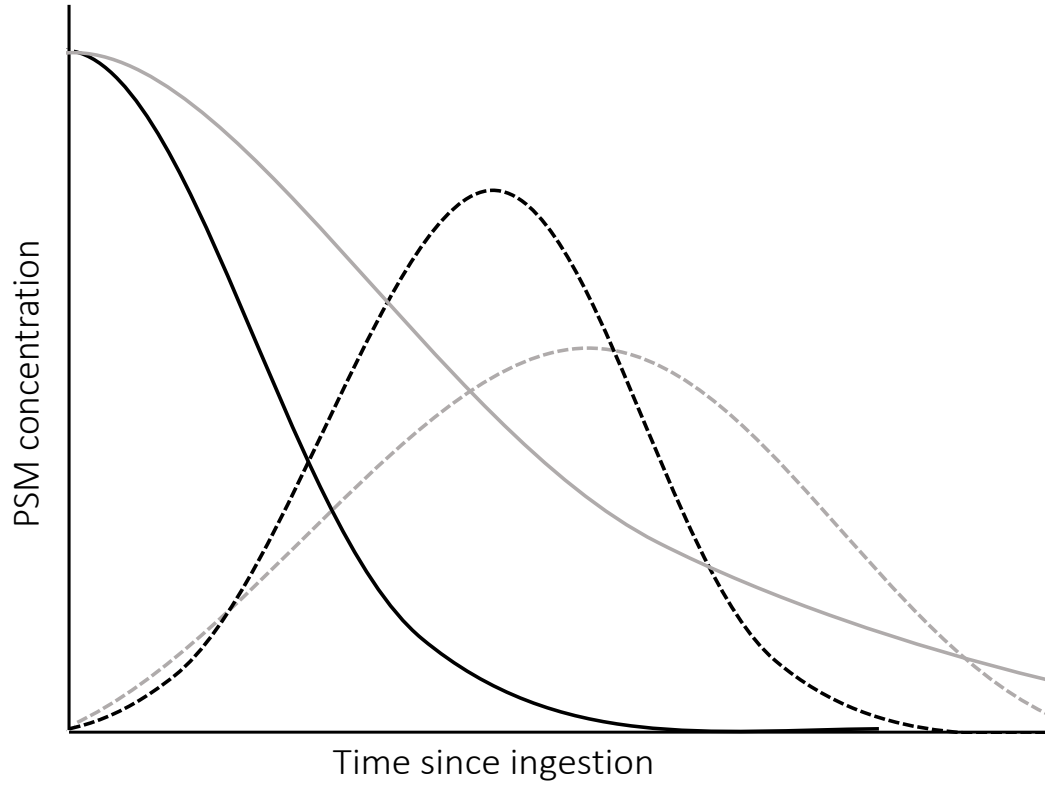

Figure 4. Schematic representation of how acclimation to different thermal environments could alter pharmacokinetics of PSMs ingested by herbivores. Solid lines indicate the concentration of a PSM in the gut of a cool (black) and warm (grey) acclimated animal. The PSM is absorbed from the gut more slowly in the warm acclimated animal. This results in a lower and longer blood PSM concentration in the warm acclimated (grey dashed line) compared to the cool acclimated animal (black dashed line).

Another possible avenue for further research is the interplay between nutrition, thermoregulation, and water conservation, which may also trade off with excretion of breakdown products. Water maintains the fluid volume of the blood, is used for

evaporative cooling and is the vehicle for excretion of breakdown products from both nutrients and PSMs. In eucalypt folivores, water is taken in primarily with food. Thus, any factors constraining food intake are also likely to affect the intake of water, and its availability for thermoregulation. Equipment limitations prevented me from accurately measuring water intake or loss during the experiments in my thesis. Therefore, it may/would be interesting/beneficial/useful to test the way in which water intake interacts with nutritional demands, nutritional limitations and with thermoregulation, to influence food intake. For example, do animals who gain water from their food select food with more water when ambient temperatures are warm, and how does this impact their nutrient intake?

Field based

Since wild herbivores are faced with a diverse nutritional landscape and constantly changing thermal environment, there is a wealth of avenues for future research in field-based studies. For example, long term data sets could be used to determine seasonal differences, or how daily temperature fluctuations impact intake at fine scales. In chapter 5 I show one way in which this type of question can be investigated in the field, namely monitoring the length and location of meals using a combination of radio- and audio-telemetry. Another method used for the detailed study of feeding by wild herbivores is habituating animals to human presence, in particular primates, and observing feeding rates directly (Rothman, Raubenheimer & Chapman 2011). Both of these methods are labor intensive, and any improvement in the ease of data collection would no doubt be welcome. It would also be useful to couple continuous monitoring of feeding behavior and ambient temperature with simultaneous data on body temperature to track whether animals store body heat or to what degree they allow body temperature fluctuations and if this is correlated with diet. Microelectronic technologies for continuous monitoring of the internal environment have advanced significantly in recent years, so that physiological parameters could be measured via implanted data loggers. This may soon prove to be a more practical, and hopefully also economic, approach to studying nutrition and thermoregulation in wild herbivores. The disadvantage is that it requires, at least initially, capture and implantation of the device. Interference with wild populations in this way will vary in its practicality so should be considered in a case by case manner.

Concluding remarks

Ultimately the aim of understanding temperature effects on feeding and PSM tolerance in herbivores is about understanding how climate change will impact free-ranging herbivores. Feeding is a fundamental requirement for animals, making access to the right food essential for their success. Through work such as that presented in this thesis we can hope to better incorporate the dynamic relationship between temperature and nutrition into conservation efforts.

References

Chillistone, S. & Hardman, J.G. (2017) Factors affecting drug absorption and distribution. *Anaesthesia & Intensive Care Medicine,* **18,** 335-339.

Christopherson, R.J. & Kennedy, P.M. (1983) Effect of the thermal environment on digestion in ruminants. *Canadian Journal of Animal Science,* **63,** 477-496.

DeGabriel, J.L., Moore, B.D., Foley, W.J. & Johnson, C.N. (2009) The effects of plant defensive chemistry on nutrient availability predict reproductive success in a mammal. *Ecology,* **90,** 711-719.

DeGabriel, J.L., Moore, B.D., Marsh, K.J. & Foley, W.J. (2010) The effect of plant secondary metabolites on the interplay between the internal and external environments of marsupial folivores. *Chemoecology,* **20,** 97-108.

Frye, G.G., Connelly, J.W., Musil, D.D. & Forbey, J.S. (2013) Phytochemistry predicts habitat selection by an avian herbivore at multiple spatial scales. *Ecology,* **94,** 308-314.

Guo, S.-T., Hou, R., Garber, P.A., Raubenheimer, D., Righini, N., Ji, W.-H., Jay, O., He, S.-J., Wu, F., Li, F.-F. & Li, B.-G. (2018) Nutrient-specific compensation for seasonal cold stress in a free-ranging temperate colobine monkey. *Functional Ecology,* **32,** 2170-2180.

Johnson, J., Minson, C. & Kellogg Jr., D. (2014) Cutaneous vasodilator and vasoconstrictor mechanisms in temperature regulation. *Comprehensive Physiology*, **4(1)**, 33-89. DOI: 10.1002/cphy.c130015.

Lawler, I.R., Foley, W.J. & Eschler, B.M. (2000) Foliar concentration of a single toxin creates habitat patchiness for a marsupial folivore. *Ecology,* **81,** 1327-1338.

Lawler, I.R., Foley, W.J., Pass, G.J. & Eschler, B.M. (1998) Administration of a 5HT3 receptor antagonist increases the intake of diets containing *Eucalyptus* secondary metabolites by marsupials. *Journal of Comparative Physiology B,* **168,** 611-618.

Mitchell, M.A. & Carlisle, A.J. (1992) The effects of chronic exposure to elevated environmental temperature on intestinal morphology and nutrient absorption in the domestic fowl (*Gallus domesticus*). *Comparative Biochemistry and Physiology A,* **101,** 137-142.

Pang, K.S. (2003) Modeling of intestinal drug absorption: roles of transporters and metabolic enzymes. *Drug Metabolism and Disposition,* **31,** 1507-1519.

Provenza, F.D., Ortegareyes, L., Scott, C.B., Lynch, J.J. & Burritt, E.A. (1994) Antiemetic drugs attenuate food aversions in sheep. *Journal of Animal Science,* **72,** 1989-1994.

Provenza, F.D., Villalba, J.J., Dziba, L.E., Atwood, S.B. & Banner, R.E. (2003) Linking herbivore experience, varied diets, and plant biochemical diversity. *Small Ruminant Research,* **49,** 257-274.

Rothman, J.M., Raubenheimer, D. & Chapman, C.A. (2011) Nutritional geometry: gorillas prioritize non-protein energy while consuming surplus protein. *Biology Letters,* **7,** 847-849.

Villalba, J.J. & Provenza, F.D. (2005) Foraging in chemically diverse environments: Energy, protein, and alternative foods influence ingestion of plant secondary metabolites by lambs. *Journal of Chemical Ecology,* **31,** 123-138.

White, R.G. (1983) Foraging patterns and their multiplier effects on productivity of northern ungulates. *Oikos,* **40,** 377-384.

Wilkinson, G.R. (1975) Pharmacokinetics of Drug Disposition: Hemodynamic Considerations. *Annual Review of Pharmacology,* **15,** 11-27.

Youngentob, K.N., Wallis, I.R., Lindenmayer, D.B., Wood, J.T., Pope, M.L. & Foley, W.J. (2011) Foliage chemistry influences tree choice and landscape use of a gliding marsupial folivore. *Journal of Chemical Ecology,* **37,** 71-84.